Neuroprotective properties of Phytoestrogens

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Women make up nearly two thirds of total Alzheimer’s cases in the United States. It has been speculated that the loss of endogenous estradiol during menopause is, at least in part, what renders the post-menopausal brain more vulnerable to the effects of aging and Alzheimer's Disease. While hormone therapy can potentially thwart some of the undesirable consequences and increased risks associated with menopause, women are increasingly rejecting hormone therapy and seeking alternative therapy. There is a strong interest in phytoestrogens as an alternative to traditional hormone therapy. Phytoestrogens are naturally occurring estrogen like compounds derived from plants which have been shown to have a variety of health benefits. Their effects in the brain however are not fully understood. It was my goal to evaluate the effect of phytoestrogens on brain cells as it relates to neuroprotection. We initially assessed the ability of genistein, the most abundant phytoestrogen found in soy, to protect brain cells against age-associated insults in vitro using the hippocampal cell line (HT22 cells), a cortical cell line (HCN-1A cells), and primary slice cultures of the cerebral cortex. The results of these experiments were such that genistein was protective in the explant model and HCN-1A cells, but not in the HT22 cells suggesting that certain key players must be present for genistein to elicit neuroprotective effects. Based on the known estrogen receptor (ER) profiles for the models used in our study, we hypothesized that ER profiles may dictate the effects of
phytoestrogens on brain cells. As such, we evaluated male and female C57/Bl6 mice at 3
different ages for ER expression profile and the effects that a phytoestrogen diet had on
BDNF, used in this study as a surrogate marker of neuroprotection. Results showed that
phytoestrogens' effects on the brain differ between the cortex and the hippocampus and
are dependent upon the sex of the animal and age at which the diet was initiated. From our
results we have proposed a mechanism by which phytoestrogens differentially elicit their
effects in the brain. The data presented herein provides valuable insight into
phytoestrogens' effects on the brain.
THE PROTECTIVE EFFECTS OF PHYTOESTROGENS

DISSERTATION

Presented to the Graduate Council of the
University of North Texas Health Science Center at Fort Worth
In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

By

Courtney Anne Brock, B.S.

Fort Worth, Texas

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CHAPTER I

INTRODUCTION

Estrogen was long thought to be a hormone mostly limited to the female reproductive system. In recent years, however, it has become clear that estrogen’s actions reach far beyond the confines of reproduction. Estrogen has a critical role in lung function, metabolism, bone health, cardiovascular function and brain function (1-6). The role of estrogen in brain health is of particular interest considering the fact that estrogen levels decrease rapidly in the menopausal woman and that women make up nearly two thirds of total Alzheimer’s cases in the United States (7). It has been speculated that this rapid decline in estrogen may be, at least in part, what renders the postmenopausal female brain more vulnerable to the effects of aging and Alzheimer’s disease (AD) (8).

There are a variety of changes that occur as the brain ages. Among these are a decrease in cortical dendritic spines, an increase in oxidative stress within the brain, and a decrease in long term potentiation (LTP) (9-13). Estrogen has been shown in many studies to be neuroprotective against age-associated insults (14, 15). Structurally, estrogen is classified as a steroid with a phenolic A ring. The phenolic A ring gives estrogen
antioxidant properties by acting as an electron donor and a free radical scavenger (16, 17). In addition to being a direct antioxidant, estrogen has been shown to upregulate antioxidant enzymes (18). Estrogen is also known to enhance LTP via activation of estrogen receptor (ER) beta and therefore improve memory (19). Other studies have also reported on estrogen’s ability to protect against brain aging as supported by the capacity of estrogen to improve scores on verbal and memory tasks in post-menopausal women and to improve spatial memory in rodent models (20-23).

Because of the potential for estrogen intervention to prevent or lessen the effects of aging on the brain, estrogen therapy is often a consideration for middle-aged women, though from the standpoint of the Food & Drug Administration (FDA), the only approved indication for use of postmenopausal estrogen therapy is to treat menopausal symptoms that include the hot flash. Estrogen therapy however is not without concern, as its use does carry certain health risks. For example, estrogen promotes cell proliferation and is involved in the development of some hormone responsive cancers such as breast and ovarian cancer (24). In 2002, the clinical trials of the Women’s Health Initiative (WHI) were ended early due to a perceived increased risk of hormone therapy on myocardial infarction, stroke, and breast cancer. In 2006, an updated analysis showed that estrogen-alone replacement did not increase the risk for breast cancer (25). However, women still remain cautious when it comes to hormone therapy citing a fear of cancer as their primary reason for declining hormone therapy (26).

For these reasons women are increasingly seeking alternatives to hormone therapy. There is a strong interest in phytoestrogens, which are plant-derived, estrogen like compounds with effects similar to mammalian estrogens. Phytoestrogens are often
considered by the general public as a natural and safer alternative to the synthetic, conjugated estrogens used in hormone therapy. Phytoestrogen intake typically occurs through one’s diet since phytoestrogens are found in abundance in soy and soy-based products. However they are also available over the counter in the form of phytoestrogen supplements or soy supplements. Soy supplements are one of the top ten herbal supplements taken in the United States with an estimated 1.7% of the population consuming them (27). They are generally marketed for relief of menopausal symptoms and as a cholesterol-lowering herb. The components of soy that are thought to mediate such beneficial effects are the phytoestrogens. Examples of the beneficial effects of phytoestrogens include such promising effects of lowering the risk of certain cancers (28, 29), lowering both total and LDL cholesterol levels (30-32), and relieving menopausal hot flashes (30, 33, 34). At this point, however, we still have limited knowledge on how phytoestrogens affect the brain, and which specific phytoestrogen components are implicated. This poses a problem since soy supplements are sold over the counter and do not require testing or approval of any kind by the FDA. With this kind of unrestricted access, it is important that we work to better understand phytoestrogens and their effects on the brain.

Estrogen receptors

Because of their structural similarity to estrogen, phytoestrogens have the ability to bind to the ER (35). The classical ER exists in two subtypes: ER-alpha and ER-beta. Once thought to exist exclusively at the nucleus where it functions as a transcription factor of estrogen responsive genes, it has more recently been shown that ER-beta is localized
primarily at the mitochondria (36) and that both ERs can localize to the plasma membrane (37-39). Their localization to the plasma membrane and mitochondria would suggest that activation of ER can result in cellular responses other than gene transcription. Current research supports this fact as estrogens have been shown to initiate rapid signaling at the plasma membrane through activation of ER-alpha (40) and also affect mitochondrial function (41). ERs are widely distributed throughout the tissues of the body. In addition to reproductive tissue, ERs have been noted to be present in bone, heart, kidney, and lung among other tissues (42-45). Of most relevance to this study is the documented presence of ER-alpha and ER-beta in the brain (46). It is their presence in the brain that we suspect influences how phytoestrogens affect brain function.

The biology of phytoestrogens:

There are three classes of phytoestrogens; isoflavones, lignans, and coumestans. Of the three types, isoflavones are the most abundant in soybeans. Genistein and daidzein are the two major isoflavones. Although these phytoestrogens are non-steroidal in structure, they possess a phenolic ring which allows them to bind to the estrogen receptor, albeit with much lower affinity than estradiol (the biologically most potent and prevalent estrogen) itself (35). Overall, phytoestrogens bind with higher affinity to ER-beta (35). The isoflavone genistein, for example, has a 20-fold higher affinity for ER-beta than ER-alpha (47). By activating the ER, phytoestrogens exert estrogen like effects by initiating transcription of estrogen responsive genes (35, 48). However, in an estrogen rich environment, phytoestrogens such as genistein act as an anti-estrogen by competitive antagonism (49). Possessing both agonistic and antagonistic properties of the ER is a desirable quality since
estrogen can be very beneficial in some organs such as the brain but detrimental in certain disease states such as breast cancer. Genistein does not only work on the ER; at higher concentrations than those that activate the ER, genistein (but not daidzein) causes an inhibition of tyrosine kinase activity (50). This can disrupt intracellular signaling and be fatal to a cell. It has been suggested that this is the mechanism for genistein's anticarcinogenic effect. Of note, these levels of genistein are attainable through dietary consumption (51).

Known mechanisms of actions for phytoestrogens:

There are several mechanisms by which phytoestrogens can be cytoprotective. For one, phytoestrogens have been implicated as antioxidants (52, 53). As an antioxidant, phytoestrogens can decrease the accumulation of reactive oxygen species within a cell thereby protecting it from further cellular damage. Alternatively, phytoestrogens can bind to the ER. Activation of the ER by phytoestrogens could lead to the same neuroprotective signaling cascades initiated by estradiol when it binds to the ER. The binding of estradiol to the ER in the brain has been shown to activate the mitogen-activated protein kinase (MAPK) signaling pathway (54). Activation of the MAPK pathway can also lead to the activation of transcription factors and therefore increase gene transcription. In fact, phytoestrogens have been shown to increase the expression of estrogens responsive genes (55). In addition to activating the MAPK pathway, the binding of estradiol to the ER also activates the phosphoinositide 3-kinase (PI3K) pathway leading to increased expression of B-cell lymphoma 2 (Bcl-2) (56). An increase in an anti-apoptotic protein such as Bcl-2 can prevent neuronal apoptosis. If activation of the ER by phytoestrogens results in the same
effects as activation by estradiol, then intracellular signaling and changes in gene expression could be mechanisms by which phytoestrogens are neuroprotective.

Phytoestrogens and neurotrophins:

One factor that is upregulated by phytoestrogens and could mediate some of the effects of phytoestrogens on the brain is the neurotrophin, brain derived neurotrophic factor (BDNF) (57, 58). Neurotrophins are growth factors that exist in two forms. They are synthesized as pro-peptides and can then be cleaved by convertases into a mature form (59). The pro-forms of all neurotrophins bind preferentially and with similar affinity to the p75 receptor. Once cleaved, however, the mature form binds preferentially to tropomyosin-related receptor kinase (trk) receptors of the tyrosine kinase family. Mature BDNF binds with high affinity to the trkB receptor whereas mature nerve growth factor (NGF) binds with high affinity to the trkA receptor. It is known that trk receptors signal through several signaling pathways, including the MAPK and PI3K pathways, to confer cell survival (60). Contrary to trk receptors, the p75 receptors contain a “death domain” within their cytosolic region which can lead to cell death upon activation by a pro-neurotrophin (61).

Neurotrophins are an important component of hormone-induced neuroprotection (62). Many studies have shown that estrogen replacement in ovariectomized rats elicits an increase in hippocampal BDNF (63-66). This may occur through a genetic mechanism where activated ER translocates to the nucleus and binds to the promoter region of the BDNF gene to regulate its transcription. In fact, it has been shown that the gene that encodes BDNF contains a sequence similar to the estrogen response element located on
many estrogen responsive genes (67). It is therefore possible that by activating the ER, genistein can modulate neurotrophin levels within the cell. In fact, data show that phytoestrogens can modulate neurotrophins, however they seem to do so in a sex-dependent manner. Female rats fed a diet rich in phytoestrogens show an increase in BDNF mRNA whereas male rats fed a diet rich in phytoestrogens show a decrease in BDNF mRNA (57, 58).

Neurotrophin levels have been linked to cognition such that higher expression of neurotrophins correlates with improved cognitive function (68). Alterations in neurotrophins may at least partially underlie the cognitive dysfunction seen in AD. In fact, post-mortem brain analysis reveals that brains of Alzheimer’s patients and those with mild cognitive impairment (MCI) show reduced levels of trkA receptors and an increase in pro-NGF (69). The neurodegeneration and/or decrease in synapses found in brains from patients with AD and MCI are consistent with the death-promoting effects that we know pro-neurotrophins to have. Neurotrophins have been more directly shown to modulate cognitive function by using viral vector technologies to introduce BDNF into the rat hippocampus. These studies found that the introduction of BDNF caused improved reference memory (70). Neurotrophins can influence cell viability through several signaling pathways. Among such pathways is the PI3K signaling pathway which when activated phosphorylates proteins such as forkhead box O3 (FOXO3) and bcl2-associated death promoter (BAD) (71). When FOXO3 is phosphorylated, it cannot enter the nucleus and induce apoptosis. Consequently, the cell is protected. Additionally, neurotrophins can activate the MAPK pathway which leads to (cAMP response element binding) CREB protein phosphorylation and ultimately an upregulation of pro-survival genes such as Bcl-2 (72).
Studies on phytoestrogens:

In vitro studies have shown phytoestrogens to be cytoprotective against a variety of age-associated insults (73-75). However, the studies done to date that address the effects of phytoestrogens on cognition have reported conflicting results. Data suggest that phytoestrogens may affect the brain differently in males and females and also potentially have age-specific effects. The latter may be consistent with a presumptive “window of opportunity” thought to exist for estrogen. In animal studies female rats tend to show beneficial effects from receiving phytoestrogens. Lund et al. reported that female rats on a lifelong diet high in phytoestrogens showed an improvement in reference memory. It is noteworthy that males on the same diet had an opposite response (76). In another study, female rats who consumed a long-term diet high in phytoestrogens showed improvement on radial arm maze performance (77). Furthermore, in studies where scopolamine was used to induce memory deficits in mice, soybean in the diet reversed these memory effects in young females through a direct estrogenic mechanism (78).

The effects of phytoestrogens on male rats have not been as consistent as those for the female rats. In one study, male rats on a lifelong phytoestrogen rich diet showed a decline in cognitive function relative to rats which were fed a phytoestrogen free diet (76). In another study done in male Wistar rats, the data showed that genistein can protect against Aβ-induced memory deficits (79). Human studies have resulted in the same discrepant results. In an aging study which included Japanese-American men, brain atrophy and cognitive decline were observed in older men who had higher midlife tofu consumption (80). However, in another study, consuming a high soy diet for 10 weeks resulted in improvements in short-term memory, long-term memory, and mental flexibility.
in both men and women (81). Postmenopausal women taking soy isoflavone supplements have also been shown to have higher category fluency and improved cognition (82, 83).

With so much discrepancy in the literature as to whether phytoestrogens exert beneficial or adverse effects on the brain, it is imperative to further investigate what might cause such a high degree of variation. Because phytoestrogens work, at least in part, through the estrogen receptors, differing levels of expression could explain the discrepancy. Estrogen receptor expression is different in males and females. Even within the same sex, expression levels vary across ages (84, 85). Receptor expression profiles can certainly impact the effect that phytoestrogens would have on the brain.

The studies reported herein, were aimed at increasing our understanding of the potential mechanisms associated with the effects of phytoestrogens on the brain. First, we assessed the cytoprotective effects of genistein, a major component of phytoestrogens in a variety of in vitro experimental models including HT22 cells and murine cerebral cortical explants. Only in the cortical explant model were the protective effects of genistein observed. In considering why only the explants would respond to genistein we proposed that differential expression of ERs could underlie the different responses. In fact, through analysis of mRNA expression using RT-PCR, we determined that the explant model was the only model to express both ER-alpha and ER-beta. Consequently we developed a working hypothesis that stated that only in models where both ER-alpha and ER-beta are expressed would phytoestrogens (genistein, in particular) be able to elicit protective effects. Based on this hypothesis, we looked at expression levels of ER in the cortex and hippocampus of mice and assessed the influence of phytoestrogen supplementation in young, middle-aged, and old male and female mice on surrogate markers of neuroprotection, namely BDNF. This
type of analysis allows us to make the distinction between the effects that phytoestrogens have in the male and female rodent brain and how those effects differ when treatment is initiated at various ages. Results show that ER-alpha and ER-beta mRNA is present in males and females at all age groups. In females, ER-alpha expression decreased with age in the hippocampus but not in the cortex. ER-beta expression in the female remains mostly unchanged with an exception of a middle-aged spike in the cortex. While age does not change expression levels of ER-beta in the males in either brain region, ER-alpha decreases with age in the cortex and increases with age in the hippocampus. The neurotrophic effects of phytoestrogens are also sex and age dependent in the cortex but not the hippocampus. With respect to BDNF, phytoestrogen supplementation led to a higher overall BDNF protein level in the hippocampi of male and female mice regardless age. In the cortex however, phytoestrogen treatment resulted in an increase in BDNF only in the young and middle-aged females whereas no such increase occurred in young or middle-aged males. In fact when the supplementation was initiated in the old male mice, the result was a decrease in BDNF.

This research establishes that while phytoestrogens have the potential to be neurotrophic and neuroprotective, whether they do so or not depends on factors such as one’s age and sex. Understanding how phytoestrogens affect the brain and the conditions under which such effects are obtained is valuable information to have as more and more people take interest in soy and phytoestrogens as a component of a healthy diet.
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CHAPTER II

GENISTEIN’S NEUROPROTECTIVE PROPERTIES IN IN VITRO MODELS OF BRAIN AGING

Courtney Anne Brock and Meharvan Singh, PhD

ABSTRACT

Phytoestrogens are naturally occurring estrogen like compounds that are found in plants and are abundant in soy and soy-based products. As results of the Women’s Health Initiative have raised concerns about the safety and efficacy of conventional estrogen therapy in reducing cardiovascular disease or dementia, the demand for suitable alternatives has emerged. One possible candidate that has been considered is phytoestrogens, which can not only mimic various aspects of estrogen biology (through their effects on estrogen receptors), but do not appear to be associated with the same risk of certain cancers (such as breast cancer), or may even exert a protective effect. While phytoestrogens are known to have many health benefits throughout the body, their effects in the brain remain unclear. Given their structural similarity to estradiol (E2), the biologically most prevalent and potent estrogen in pre-menopausal women, it is hypothesized that they share estrogen’s neuroprotective properties. However, the results
of clinical studies which have tested genistein's effects on memory and cognition have varied. We have thus set out to test the hypothesis that genistein is neuroprotective by studying its ability to protect various models of brain cells against age-associated insults. Using mouse cortical explants, the HT22 mouse hippocampal cell line, and the HCN-1A human cortical cell line, we determined if genistein, a major component of phytoestrogens was protective against the excitotoxic and pro-oxidative stress-inducing agent, glutamate and the pro-oxidative stress-inducing agent H2O2. The data revealed that while genistein was protective against the glutamate insult in the cortical explant model and the HCN-1A cell line, it did not protect the HT-22 cell line from glutamate-induced cell death. These data support our hypothesis that genistein is neuroprotective, but only within certain cellular contexts. As such, we infer that certain criteria/cellular mediators must exist for genistein to elicit a protective effect.
INTRODUCTION

Many people across the globe consume soy and soy products on a regular basis either as a part of their natural diet or in the form of supplementation. Soy contains a high concentration of estrogen like compounds known as phytoestrogens. Phytoestrogens are plant-derived compounds which are structurally similar to estrogen and can therefore bind to and activate the estrogen receptor (ER) causing estrogenic effects (1-3).

Genistein is the most abundant phytoestrogen found in soy. It is present at nearly 5ug/g of soybean and at a much higher concentration of 229ug/g in fermented soybean products such as miso (4). It is therefore present in the body of many people who consume soy on a regular basis and in people who consume soy or isoflavone supplements (5). In general, genistein is considered to be beneficial to one’s health. It has shown to be effective at lowering the risk of certain cancers (6, 7), lowering both LDL and total cholesterol levels (8-10), and relieving menopausal hot flashes (8, 10, 11). Because genistein is known to have estrogenic effects in the periphery (12), in recent years there has been interest in whether or not it can mimic estrogen’s protective effects in the brain. If genistein mimics estrogen’s neuroprotective properties, it could potentially serve an important function in maintaining brain health after the menopause when endogenous levels of estrogen decrease in women.
In vitro studies on the cytoprotective effects of genistein have shown that it is able to protect against a variety of insults in neuronal models including glutamate toxicity (13), oxidative stress (14), and beta-amyloid toxicity (15). Because genistein can bind to and activate the ER (1, 2), it can potentially protect through ER-mediated neuroprotective mechanisms such as enhancement of gene transcription for genes such as brain derived neurotrophic factor (BDNF) or elicit neuroprotective cell signaling through activation of the ER. Activation of such signaling pathways as the MAPK and PI3K pathways can, in turn, lead to an increase in the anti-apoptotic protein Bcl-2 (16, 17).

Based on results of in vitro studies, the assumption could be made that phytoestrogens are capable of improving brain function through neuroprotection at the cellular level. However, the clinical studies which have investigated the effects of phytoestrogens on cognition have not provided a clear understanding of whether or not phytoestrogens improve cognitive function. Some studies have shown an association between higher soy consumption and brain atrophy and cognitive decline later in life (18) whereas other clinical studies have shown improved cognitive function as a result of soy consumption (19-21). With such conflicting results, it becomes important to study the effects that phytoestrogens have on brain cells and the mechanism by which they influence cell viability. Understanding how phytoestrogens affect brain cells will help to predict whether they will be beneficial to cognitive function or not, and therefore, will provide insight into whether phytoestrogens may be a suitable alternative to traditional hormone therapy. In order to better characterize the cytoprotective potential of phytoestrogens, we have
used a variety of \textit{in vitro} models to test the hypothesis that genistein is neuroprotective against age-associated insults.
MATERIALS AND METHODS

**Cell culture and reagents:** HT22 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cells were maintained in a humidified incubator at 37°C containing 5% CO₂. 24 hours before treatment, media was replaced with DMEM supplemented with 10% charcoal-stripped fetal bovine serum and 1% penicillin-streptomycin. Genistein (100nM and 500nM) was applied 24 hours before glutamate (2mM) treatment. Cells were incubated with glutamate for 20 hours before measuring cell viability. In experiments using tert-butyl hydrogen peroxide (30uM), the insult was applied to cells for 5 hours. Glutamate and tert-butyl hydrogen peroxide solution were obtained from Sigma-Aldrich (St. Louis, Missouri). Human cortical neurons (HCN-1A) were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were differentiated by changing media to that which contains 25ng/ml nerve growth factor (NGF), 0.5mM dibutyryl cyclic AMP (dbcAMP), and 0.5mg/ml 3-isobutyl-1-methylxanthine (IBMX) for 3 days. After differentiation, cells were treated with genistein (10nM, 100nM, 500nM) for 24 hour before a 24 hour treatment with glutamate (0.1mM).

**Tissue Culture:** Cerebral cortical explants were derived from postnatal day 3 (P3) C57Bl/6J mice. Each slice is a 360-um-thick hemicoronal slice of the frontal and cingulate cerebral cortex, maintained in culture (three hemicoronal slices per tube) on glass
coverslips which have been pre-coated with rat tail collagen-coated/poly-L-lysine. The cultures were maintained in maintenance medium (25% heat inactivated gelded horse serum (Sigma, St. Louis, Missouri), 22.5 % Hank’s balanced salt solution (Mediatech. Inc., Herndon, Virginia), 50% minimum essential medium Eagle MEM (Sigma), 5.25 mg/ml of D(+)-glucose (Sigma), 2 mM L-glutamine (Mediatech. Inc.), 50 ug/ml L-ascorbic acid (Sigma) supplemented with 2 nM 17-β estradiol (Steraloids, Newport, Rhode Island). On the 6th day in vitro, media was exchanged for estrogen free media and the cultures were pretreated experimentally with genistein on day 7 in vitro. 24 hours after pretreatment, glutamate (15mM) was added to the cultures and incubated for 6 hours after which tissue was harvested and media used for an LDH assay.

**Lactate Dehydrogenase (LDH) Assay:** Media derived from treated cells was used in a fluorometric assay (CytoTox-One Homogenous Membrane Integrity Assay Kit from Promega) which measures the amount of LDH released from dead or dying cells, a reflection of compromised plasma membrane integrity. In short, in a black, clear bottom, 96-well plate, 100uL of CytoTox-One reagent was added to 100uL of conditioned media and incubated for 10 minutes. After 10 minutes, 50uL of Stop Solution is added to each well to terminate the enzymatic reaction and fluorescence was measured with the aid of a Filtermax F5 plate reader (Molecular Devices, Sunnyvale, CA) using the following parameters: Excitation wavelength - 560 nm; Emission wavelength - 590 nm.

**Calcein Assay:** Calcein AM from Anaspec (Fremont, California) was used according to an established protocol to discern cell viability. In short, cells were plated in black, clear bottom 96 well plates on the day preceding the experiment. After experimental treatments
were complete, the media was removed from the cells and washed with 60uL of phosphate buffered saline (PBS). After removal for the PBS, 60uL of 2mM calcein was added to each well and incubated for 30 minutes at 37°C with 5% CO₂. After incubation, the fluorescence levels were read at 485 nm excitation and 535 nm emission wavelengths using a plate reader.

**Statistical Analysis:** Calcein AM data and data from cellular experiments measuring LDH from a 96 well plate were obtained from a minimum three independent experiments (N=4 for each experiment). Data was averaged and analyzed using One-way ANOVA analysis followed by a Tukey’s post-hoc analysis (GraphPad Software, San Diego, California). Data from cortical explants was obtained from a minimum of 3 independent experiments. Data was averaged and analyzed using One-way ANOVA analysis followed by a Tukey’s post-hoc analysis (GraphPad Software, San Diego, California).
RESULTS

Genistein fails to protect HT22 cells from H$_2$O$_2$ induced cell death

Increased cellular oxidation is a known consequence of brain aging (22). Here, H$_2$O$_2$ is used as an agent that elicits cell death by inducing oxidative damage within the cells. To test whether genistein is able to rescue hippocampal HT22 cells from H$_2$O$_2$ induced cell death, genistein, was co-applied with H$_2$O$_2$ (30uM) for 5 hours. This concentration and time course were chosen based on time-course and concentration-response experiments demonstrating that a 30uM treatment for 5 hours produces 25-30% cell death. We consider this level of cell death, optimal for the assessment of protection, as the chosen conditions do not injure cells to such an extent to which they are incapable of being rescued. The calcein AM assay was used to measure the live cells remaining after the treatment. Results show that a co-application of genistein was ineffective at preventing or reducing H$_2$O$_2$ induced cell death (Fig. 1).

Pretreatment of HT22 cells with genistein failed to protect HT22 cells from H$_2$O$_2$ induced cell death

The data described above suggested that genistein was unable to protect HT22 cells from H$_2$O$_2$ induced cell death when co-applied with the insult (Fig.1). We proposed that specific cellular events needed to occur prior to the insult in order for genistein to elicit a protective effect. To test this, genistein was applied to the cells 1 hour (Fig.2) and 24 hours
(Fig. 3) prior to H$_2$O$_2$ (30uM) exposure. H$_2$O$_2$ exposure lasted 5 hours, after which the calcein AM assay was used to measure the live cells remaining after the treatment. Results show that neither a 1 hour nor 24 hour incubation with genistein prevented or reduced the cell death caused by H$_2$O$_2$ exposure. It is noteworthy, however, that a 1 hour pretreatment (Fig. 2) and a 24 hour pretreatment (Fig. 3) with 17-β estradiol (serving as our positive control) significantly protected these cells from H$_2$O$_2$ toxicity (p<0.05 versus H$_2$O$_2$).

**HT22 cells are not protected from glutamate induced cell death by genistein applied twenty four hours prior to insult**

Because glutamate is another common age-associated insult in the brain, we determined whether the lack of effectiveness of genistein in the above experiments was attributed to the nature of the insult. As such we determined if genistein could mitigate the toxic effects of glutamate, a compound that can elicit cell death through both excitotoxic and pro-oxidant means. In this experiment, HT22 cells were pretreated with genistein for 24 hours prior to a 20 hour exposure to 2mM glutamate. The concentration and time point chosen for glutamate exposure in this experiment were based on time-course and concentration-response experiments in which we determined that a 20 hour treatment of 2 mM glutamate resulted in 25-30% cell death. After the glutamate exposure, a calcein AM assay was used to assess cell survival. Results show that genistein was unable to protect HT22 cells against glutamate-induced cell death (Fig. 4).
Genistein (500 nM) is effective at reducing glutamate induced cell death in HCN-1A cells

HT-22 cells do not express ionotropic glutamate receptors, and as such the consequence of glutamate treatment is expected to result in cell death primarily through a mechanism that involves an increase in oxidative stress accompanied by a reduction in intracellular anti-oxidant defenses (glutathione, in particular). As such, we suggested that genistein may only be protective in cells where glutamate is excitotoxic. In fact, hormones, such as progesterone, have previously been shown in our lab to reduce cell death in neuronal tissues that has resulted from an excitotoxic insult (23). Glutamate is used here as an inducer of excitotoxicity in HCN-1A cells. Genistein was applied to the cells for 24 hours prior to the glutamate insult. An LDH assay was used as an indicator of cell death at the end of the treatment. Results show that genistein (500nM) effectively completely reduced the amount of cell death caused by glutamate (a 99% reduction) in the HCN-1A cells (p<0.05 versus glutamate) (Fig. 5).

Genistein (100 nM) is effective in reducing glutamate induced cell death in primary slice cultures (explants) of the cerebral cortex

The mouse cerebral cortical explant model is one that contains intact neuronal and glial interactions within each slice, has been shown to express both ER-alpha and ER-beta, and has been used by our lab in the past to investigate mechanisms of neuroprotection (23). The ability of genistein to protect against a glutamate insult was tested in this model. Genistein was applied 24 hours prior to glutamate application. Explants were then exposed to 15mM glutamate for 6 hours, a concentration and time point used previously in our
laboratory for neuroprotective studies (23). An LDH assay was used as an indicator of cell death at the end of the treatment. Results show that genistein (100nM) was able to significantly reduce the amount of cell death by 62% of that caused by glutamate in the cerebral cortical explants (p<0.05 versus glutamate) (Fig. 6).

The neuroprotective effects of genistein are abolished the ER antagonist, ICI 182 780

Because genistein can bind to and activate the ER, we conducted an experiment to determine if genistein’s neuroprotective effects in the explants were dependent upon ER activation. To do this we applied the ER antagonist, ICI 182 780 (100nM) 15 minutes prior to the application of genistein to effectively block both ER-alpha and ER-beta. 100nM ICI 182 780 has been reported in the literature to effectively block phytoestrogens from both ER-alpha and ER-beta (24, 25), and is approximately 300 times the IC₅₀ for ER-alpha or –beta (26). Genistein was then applied to the cultures 24 hours prior to glutamate treatment. An LDH assay was used as an indicator of cell death at the end of the treatment. Results show that in the presence of the ICI 182 780, genistein’s protective effects against glutamate-induced cell death in the explants is abolished (p<0.05 versus glutamate) (Fig. 7).
Coapplication of estrogen/genistein on HT22 cells

Untreated 2O2H

100nM Genistein+H2O2

500nM Genistein+H2O2

1uM Genistein+H2O2

1uM Estrogen+H2O2

5 hour treatment of 30uM H2O2

*: statistically different from H2O2

Live Cells (% untreated control)
Figure 1. Co-application of genistein with H$_2$O$_2$ does not protect against H$_2$O$_2$ induced cell death in HT22 cells. HT22 cells were plated in 96 well plates at 5,000 cells/well on the day prior to genistein application. 24 hours after plating the cells the media was removed and replaced with media containing 30uM H$_2$O$_2$ and either 100nM genistein, 500nM genistein, 1uM genistein, or 1uM E$_2$. The cells were incubated for 5 hours after which the media was removed and a calcein AM assay was performed. Results from the calcein AM assay show that neither genistein nor E$_2$ was able to protect the cells against an H$_2$O$_2$ induced cell death when co-applied together. Data are derived from 4 independent experiments. (# denotes a statistical significance from untreated control, p<0.05, as measured by a One Way ANOVA followed by tukey’s post hoc analysis).
1 hour pretreatment of HT22 cells

Untreated

2O2H

100nM Genistein+H2O2

500nM Genistein+H2O2

1uM Genistein+H2O2

1uM Estrogen+H2O2

5 hour treatment with 30uM H2O2

Live Cells (% untreated control)

untreated  H2O2  100nM Genistein+H2O2  500nM Genistein+H2O2  1uM Genistein+H2O2  1uM Estrogen+H2O2

*  #  #  #  #  *  #
Figure 2. A 1-hour pretreatment of genistein does not protect HT22 cells from H₂O₂-induced cell death. HT22 cells were plated in 96 well plates at 5,000 cells/well. 24 hours after plating, the media was removed and replaced with media containing 100nM genistein, 500nM genistein, 1uM genistein, or 1uM E₂. After one 1 hour, media was again removed and replaced with media containing 30uM H₂O₂ and either genistein or E₂. The cells were incubated for 5 hours before performing a calcein AM assay. Results show that genistein did not protect against H₂O₂-induced cell death at the tested concentrations however 1uM E₂ was significantly protective. Data are derived from 4 different experiments. (# and * denote a statistical significant difference compared to untreated control and H₂O₂ treated cells respectively, p<0.05, as measured by One way ANOVA followed by a tukey's post-hoc analysis).
24 hour pretreatment of HT22 cells

untreated

$\text{H}_2\text{O}_2$

$100\text{nM Genistein} + \text{H}_2\text{O}_2$

$500\text{nM Genistein} + \text{H}_2\text{O}_2$

$1\text{uM Genistein} + \text{H}_2\text{O}_2$

$1\text{uM Estrogen} + \text{H}_2\text{O}_2$

5 hour treatment with $30\text{uM H}_2\text{O}_2$

*:* statistically different from $\text{H}_2\text{O}_2$

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Live Cells (% untreated control)
Figure 3. A 24-hour pretreatment of genistein does not protect HT22 cells from H₂O₂-induced cell death. HT22 cells were plated in 96 well plates at 5,000 cells/well. 24 hours after plating, the media was removed and replaced with media containing 100nM genistein, 500nM genistein, 1uM genistein, or 1uM E₂. After 24 hours, media was again removed and replaced with media containing 30uM H₂O₂ and either genistein or E₂. The cells were incubated for 5 hours before performing a calcein assay. Results show that genistein did not protect against H₂O₂-induced cell death at the tested concentrations however 1uM E₂ was significantly protective. Data are derived from 4 independent experiments. (# and * denote a statistical significant difference compared to untreated control and H₂O₂ treated cells respectively, p<0.05, as measured by One way ANOVA followed by a tukey's post-hoc analysis).
Transform of 2mM with genistein: Transformed data

untreated Glutamate
100nM Genistein+glutamate
500nM Genistein+glutamate

Live Cells (% untreated control)
Figure 4. A 24 hour pretreatment with genistein does not protect HT22 cells against a glutamate-induced cell death. In this experiment HT22 cells were plated in 96 well plates at a density of 2,500 cells/well. 24 hours after plating the cells, the media was replaced with media containing either 100nM or 500nM genistein. 24 hours after genistein application, media was again replaced with media containing genistein and 2mM glutamate. The cells were then incubated for 20 hours after which, the calcein AM assay was performed. Results show that a 24 hour pretreatment with genistein did not prevent or reduce the glutamate-induced cell death. Data are derived from 4 independent experiments. Data are derived from 3 independent experiments. (# denotes a statistically significant difference from untreated control; p<0.05).
Figure 5. Genistein (500 nM) is effective at reducing glutamate induced cell death in HCN-1A cells. HCN-1A cells were grown in culture in 60mm petri dishes. At the onset of the experiment, media was replaced with fresh media containing 1nM E2, 10nM E2, 10nM genistein, 100nM genistein, or 500nM genistein. 24 hours later, .1mM glutamate was added to the cells and incubated for an additional 24 hours, after which the media was subjected to LDH analysis. The detergent triton-x was used here as a control for cell death as it lysed the plasma membrane. Results show that 500nM genistein is protective against glutamate-induced cell death in the HCN-1A cells. Data are derived from 3 independent experiments. (# and * denotes statistical significant difference from untreated control and glutamate treated cells, respectively, p<0.05, as measured by a One way ANOVA followed by Tukey's pot-hoc analysis).
100nM Genistein protects against glutamate-induced LDH release in male-derived cerebral cortical explants.
Figure 6. Genistein (100nM) is effective at reducing glutamate-induced cell death in the cerebral cortical explant model. Hemicoronal slice cultures (360um thickness) from the cerebral cortex of the P3 C57/Bl6 mouse were generated and maintained in roller tubes with 3 slices per tube. On day 6 in culture, the media was changed to estrogen free media. On day 7 in culture genistein (10nM, 100nM, or 500nM) was added to culture medium and incubated for 24 hours. 100nM progesterone (P4) was used as a positive neuroprotective control. 24 hours after genistein application, 15mM glutamate was added to the culture medium and incubated for 6 hours. After incubation with glutamate, media was collected and used in an LDH assay. Results show that 100nM genistein protected the explants from glutamate-induced cell death. Data are derived from 6 independent experiments. (# and * denote statistical significant difference from DMSO and glutamate treated cultures respectively, p<0.05, as measured by a One way ANOVA followed by a Tukey's post-hoc analysis).
ICI-182,780 prevents genistein induced neuroprotection.
Figure 7. ICI-182,780 prevents genistein induced neuroprotection in cerebral cortical explants. Hemicoronal slice cultures (360um thickness) from the cerebral cortex of the P3 C57/Bl6 mouse were generated and maintained in roller tubes with 3 slices per tube. On day 6 in culture, the media was changed to estrogen free media. On day 7 in culture ICI 182,780 (100nM) was added to the culture tube 15 minutes prior to genistein application. Genistein (10nM, 100nM, or 500nM) was then added to culture medium and incubated for 24 hours. 24 hours after genistein application, 15mM glutamate was added to the culture medium and incubated for 6 hours. After incubation with glutamate, media was collected and used in an LDH assay. Results show that ICI 182,780 abolished the neuroprotective effects of genistein. Data are derived from 3 independent experiments. (# and * denote statistical significance from DMSO and glutamate treated cultures respectively, p<0.05, as measured by a One way ANOVA followed by Tukey’s post-hoc analysis).
DISCUSSION

The data presented here show that genistein is capable of protecting brain cells against specific age-associated insults (Fig. 5 and Fig. 6). Interestingly, genistein was only protective in some models, which is indicative that there may be certain criteria that must be met in order for genistein to have neuroprotective effects. Such conditional neuroprotection could potentially explain why clinical studies have reported conflicting findings on the effects of phytoestrogens. Being able to determine under which conditions genistein protects could be instrumental in defining which subset of the population might benefit from soy or phytoestrogen consumption.

To better understand the conditions under which genistein will be protective, it is necessary to further analyze the properties and characteristics of the models used in this study. To begin, the HT22 cell line is a murine hippocampal cell line. Both the HCN-1A cell line and the explants were derived from cortical neurons. The HCN-1A cell line is a cortical cell line derived from human brain tissue. It is noteworthy that this cell line is no longer available through ATCC and further studies cannot be carried out using this model. The explants are also a cortical model, however one that is derived from young (perinatal) mouse pups and includes both the neurons and the glia within each slice. In our studies, the HT22 cells were not protected by genistein against either a glutamate or H$_2$O$_2$ insult (Figs. 1-4), whereas the other cellular models were protected by genistein against glutamate-
induced toxicity (Fig. 5 and Fig. 6). One explanation lies in the fundamental difference between the HT22 cells and the other models which is the origin of the cells. It is possible that there are cellular markers that exist on cortical neurons which provide a mechanism for genistein to protect which do not exist in hippocampal neurons.

Another possibility to explain the differences in protective ability seen in this study is the expression of the ER. Our lab has shown that the HT22 cell line expresses ER-alpha but not ER-beta. The explants, on the other hand express both ER-alpha and ER-beta. Because genistein is an ER-beta preferring ligand, the fact that it is absent in the HT22 cells might account for the lack of observed effect. The presence of the ER in the HCN cell line is unknown however work published by others has shown that HCN-1A cells respond to estradiol in a concentration-dependent manner (27) and are protected by naturally occurring phytoestrogens such as red clover (28). This would suggest that the ER is present in this cell line.

The requirement for ER in genistein’s protective effects is supported by the data presented here (Fig. 7). In the explants, when the ER was blocked by the receptor antagonist, ICI 182,780, genistein lost its ability to protect the cultures against a glutamate induced cell death. This supports our hypothesis that the ER must be present for genistein to protect.

Phytoestrogens are consumed by many people all over the world, either as a natural part of their diet or as supplemental phytoestrogen therapy. Without regulation by the FDA, neither the short term nor long term effects of phytoestrogens on the brain are clear. Clinical studies have been conducted but have reported conflicting findings as to whether phytoestrogens improve or worsen memory and cognition (18-21). The data presented
here hint at a possible explanation for the conflicting results of the clinical studies. The data suggest that genistein has the potential to protect brain cells against insults commonly associated with brain aging, however certain cellular and molecular entities must be present in order for genistein to effectively protect the cells from death. Establishing which molecular players are essential in genistein's neuroprotection may be the key to determining which people will benefit from phytoestrogen consumption or phytoestrogen therapy.
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CHAPTER III

THE REGULATION OF BRAIN-DERIVED NEUROTROPHIC FACTOR BY PHYTOESTROGENS IN C57BL/6 MICE: THE INFLUENCE OF AGE AND SEX

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ABSTRACT

Consuming a diet high in soy has many health benefits including its potential to improve brain function. However, studies on the effects of soy and phytoestrogens in the brain have been somewhat inconsistent leading to the need to investigate the parameters that may influence the sensitivity of the brain to the protective effects of phytoestrogens. In that respect, we have set out to investigate how phytoestrogens affect the two brain regions most closely linked to learning and memory, the cortex and the hippocampus, at different ages in both males and females. To accomplish this, the effects of phytoestrogen supplementation on a surrogate marker of cognitive function, Brain Derived Neurotrophic Factor (BDNF) in male and female mice of three different ages (6 months, 12 months, and 24 months) were evaluated. Since phytoestrogens can elicit their effects through activation of the estrogen receptor (ER), and that activation of the estrogen receptor can increase the expression of BDNF, we also investigated whether a correlation existed between ER-alpha or ER-beta (or the combination thereof) and the influence of phytoestrogen
supplementation on BDNF. Our results demonstrate that the effects of phytoestrogens on BDNF in the cortex were dependent on when the diet was initiated (i.e., at which age). Further, the pattern of regulation differed between males and females. Phytoestrogens increased BDNF protein in the hippocampus of males and females independent of when the diet was initiated. After a secondary analysis of the data we observed a relationship between ER and effects of phytoestrogens on BDNF in females. Based on this we proposed a mechanism by which phytoestrogens act to alter BDNF.
INTRODUCTION

As the brain ages, natural changes occur which include a decrease in cortical dendritic spines, an increase in oxidative stress within the brain, and a decrease in long term potentiation (LTP) which can cause cognitive decline and memory loss (1-5). These functional changes are exacerbated in disease states such as Alzheimer’s Disease (AD). Preserving brain function as one ages is critical to maintaining one’s quality of life. Phytoestrogens have been considered as a natural way to maintain or improve one’s cognitive function because of the similarities they share with estradiol, a known neuroprotective hormone.

Phytoestrogens are naturally occurring, estrogen-like compounds that are consumed by many people either through soy-derived foods or through herbal supplements. Because of their structural similarity to estrogen, many have speculated that phytoestrogens may be neuroprotective like estrogen. The clinical studies that have investigated the effects of phytoestrogens on memory and cognition, however, have reported conflicting results. Some studies have reported that soy or phytoestrogen supplementation improves cognitive function (6-8). Other studies, however, have reported an increase in brain atrophy and cognitive decline in those with higher soy consumption (9, 10). Thus, it is not yet clear how phytoestrogens affect brain function. Our laboratory has studied the neuroprotective effects of genistein, the most abundant phytoestrogen in soy and found that it does have the potential to be neuroprotective but appears to require certain molecular players in order to exert protective effects. Because phytoestrogens can bind to and activate the ER (11-13), that ER activation can increase BDNF (14, 15), and
BDNF has been attributed to improved cognition (16, 17), we believe that phytoestrogens are neuroprotective, potentially through an ER-mediated induction of neurotrophins. The caveat to this however, is that the levels of ER and BDNF change as one ages and also differ between males and females (18-20). As such, the effectiveness of phytoestrogens as a brain protectant may differ as a function of sex and age. In fact, this may at least partially explain why clinical studies have reported conflicting findings with regards to phytoestrogen supplementation. In this study we have attempted to address these age related changes and sex differences by studying the mRNA profile of ER-alpha and ER-beta and the effects that a diet rich in phytoestrogens has on BDNF mRNA and protein expression in the brains of male and female mice at a young age, a middle age, and an old age. Such information will carry a high predictive value in determining which subset of people may have improved brain function as a result of soy or phytoestrogens. Because so many people consume phytoestrogens it is essential that we establish the effects that phytoestrogens have on the brain.

Learning and memory are controlled largely by the cortex and the hippocampus. To understand a drug's effect on memory, it is crucial to study its effects in these brain regions. It is for that reason that the cortex and the hippocampus were chosen as the brain regions of interest in this study. In the previous chapter we determined that a major component of phytoestrogens, genistein, elicited neuroprotective effects in cell culture models. In the studies described below, we explored whether phytoestrogens would increase the expression of BDNF, which is used here as a potential surrogate marker of neuroprotection, and potentially, cognitive enhancing effects. The assessment of BDNF mRNA and protein was conducted in hippocampal and cerebral cortical tissue derived from male and female
C57/Bl6 mice, at three different ages. In view of the *in vitro* data presented in the previous chapter and the inconsistent effects of phytoestrogen supplementation described in the literature, we hypothesized, as a means to explain the discrepancy, that the induction of BDNF by phytoestrogens would be dependent on the expression of ERs. As such, in this study we determined whether the effects of phytoestrogen supplementation were correlated with the expression of either ER-alpha or ER-beta.
MATERIALS AND METHODS

Animals: C57Bl/6 mice were obtained from Charles River Laboratories. Male and female mice were housed separately. Test animals were started on a phytoestrogen rich diet at 6 months, 12 months, or 24 months to represent young, middle-age, and old, respectively. The animals were maintained on the diet for 5 weeks before behavioral testing (data not included) and then maintained on the diet for the duration of the behavioral testing which lasted 8 weeks resulting in a total phytoestrogen exposure of 13 weeks. After behavioral testing, the animals were euthanized.

Diet: For 13 weeks, animals were fed either a control (phytoestrogen free – 2016 from Harland teklad) diet or a test (phytoestrogen rich – 8604 from Harland teklad) diet. Both diets had similar contents with the exception of the phytoestrogen content. Genistein levels are undetectable in the phytoestrogen free diet and are reported at 190 ppm in the phytoestrogen rich diet (21). This has been reported to result in an average steady state plasma genistein level of 160 +/- 79 ng/ml (22) which falls within the range observed for humans who consume soy or supplements (23, 24).

RNA isolation and cDNA synthesis: RNA was extracted from brain tissue using the RNeasy Lipid Kit (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. RNA concentration was calculated from the measured absorbance at 260nm. 2ug of RNA was reverse transcribed into cDNA using the High-Capacity DNA Archive Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions.
**Primers and Probes for quantitative real-time RT-PCR:** PCR primers and probes for the target gene, ER alpha and ER beta and the endogenous control GAPDH were purchased as Assay-On-Demand (Applied Biosystems Inc., Foster City, CA.). The assays are supplied as 20x mix of PCR primers (900nM) and TaqMan probes (200nM). The ER-alpha (Mm00433149_m1), ER-beta (Mm0059984_m1), BDNF (Mm00432069_m1) assays contained FAM (6-carboxy-fluorescein phosphoramidite) dye label at the 5 prime end of the probes and minor groove binder and non-fluorescent quencher at the 3 prime end of the probes. The GAPDH assay (Mm99999915_g1) contained VIC-labeled probes. The assays are optimized for use on ABI Prism Sequence Detection System at the default machine settings.

**Quantitative Real-Time RT-PCR:** A reaction mixture (water, 2x quantitative PCR Master Mix (Eurogentec, Freemont, CA), and primer) was aliquoted in a 96-well plate. cDNA was added to each well. Each sample was analyzed in triplicate. A control for each sample was performed in triplicate in which GAPDH was measured. The ABI 7300 Sequence Detection System (Applied Biosystems) was used for amplification and detection. The software used for data analysis was Sequence Detection Software version 1.3 (Applied Biosystems). The RT-PCR program was as follows: 2 minute hold at 50°C (uracil-N-glycosylase) and 10 minute hold at 95°C, followed by 40 cycles of 15 seconds at 95°C (denaturation) and 1 minute at 60°C (annealing and extension). Relative changes in target gene expression were calculated using the comparative cycle threshold (Ct) method (25).
**BDNF ELISA:** To quantify BDNF protein levels in brain tissue we used the BDNF Emax® Immuno Assay Systems (Promega, Madison, WI). Briefly, a 96-well Nunc MaxiSorp polystyrene flat-bottom immunoplate was precoated with an anti-BDNF monoclonal antibody (diluted 1:1,000 in coating buffer (25mM sodium bicarbonate and 25mM sodium carbonate, pH 9.7). Unbound antibody was washed off with TBS-T buffer (20mM Tris-HCl (pH7.6), 150mM NaCl and 0.05% (v/v) Tween-20) and the plate was blocked to minimize nonspecific binding. 150ug of sample lysate (in a final volume of 100ul) was added to the plate. Each sample was run in duplicate. Two columns of the plate were reserved for the BDNF standard curve which included concentrations ranging from 7.8 to 500pg/ml. Plates, then underwent a series of washes with TBS-T after which the captured BDNF was incubated with the polyclonal anti-human BDNF antibody. Anti-Ig Y-horseradish peroxidase (HRP) was then added to the wells which binds with the polyclonal antibody. Excess was rinsed through a series of washes with TBS-T. TMBone reagent was added to each well which induces a color change which is measured using a Filtermax F5 plate reader (Molecular Devices, Sunnyvale, CA).

**Statistical analysis:** Both mRNA and protein data were obtained from a minimum of 6 independent samples and analyzed using a t-test for two group comparisons and a One-way ANOVA followed by Tukey’s post-hoc analysis for three group comparisons. (GraphPad Software, San Diego, California).
RESULTS

Expression of ER-beta but not ER-alpha mRNA increases in the middle-aged female cortex

ER-alpha has been shown to be the mediator of estradiol-induced neuroprotection in models of middle cerebral artery occlusion (MCAO)-induced injury (26), beta-amyloid toxicity (27), and glutamate toxicity (28-30). As a critical mediator of estrogen’s neuroprotective properties, the effects of estrogens (and phytoestrogens) on the brain may depend on its presence at the time of treatment. While a role for ER-alpha has been documented in the neuroprotective effects of estradiol (26-28, 30, 31), ER-beta is not excluded as a mediator of estrogen induced neuroprotection. In fact, one study has shown ER-beta to be the primary mediator of estradiol’s protective effects against excitotoxicity (32). Recently, the phytoestrogen genistein was shown to decrease apoptosis of cultured motoneurons through an ER-dependent mechanism which was associated with an increase in ER-beta (33). Because ER-beta is known to play a role in estrogen and phytoestrogen induced neuroprotection its expression at the time of administration may influence the outcome of treatment. To investigate whether ER-alpha or ER-beta levels change with age we measured the level of mRNA from the cortex of male and female mice aged 9, 15, and 27 months to represent young, middle-aged, and old mice, respectively. The control group (young) is set to 1 and results are expressed as a fold change relative to control. Results (Fig.1 top panel) show that while there were no age-related changes in ER-alpha levels, ER-beta levels showed a significant increase (87%), but only at middle-aged, (p<0.05 versus control).
A phytoestrogen rich diet suppresses BDNF in the middle-aged (but not young and old) female cortex

BDNF is a critical component of hormone induced neuroprotection (34) and has been shown to be regulated by phytoestrogens (35, 36). Having identified how ER-alpha and ER-beta mRNA expression changes with age in the male and female cortex (Fig. 1 top panel), we had a premise with which to investigate how the presence and/or relative abundance of ER-alpha and ER-beta mRNA dictates the effects that phytoestrogens have on BDNF mRNA expression in the cortex. As such we have measured how a phytoestrogen rich diet affects BDNF mRNA levels using real time RT-PCR. Mice had been initiated on a phytoestrogen rich diet at 6 months of age, 12 months of age, or 24 months of age to represent young, middle-aged and old, after which we measured BDNF mRNA levels from each age group. Results (Fig. 1 left side) show that relative to age-matched controls, the phytoestrogen rich diet only affected the BDNF mRNA levels in the middle-aged group, causing a 21% decrease, (p<0.05 versus control).

A phytoestrogen rich diet increases BDNF protein in the young and middle-aged but not the old female cortex

To use BDNF as a surrogate marker of neuroprotection, it is valuable to measure how phytoestrogens affect protein levels of BDNF. As such, we assessed how a phytoestrogen rich diet affects BDNF protein levels in mice that had initiated the diet at a young age, middle, or old age. Results (Fig. 1 right side) show that compared to age-matched controls, the phytoestrogen rich diet caused a 100% and 150% increase in the
young and middle-aged female cortex but had no effect in the old animals, (p,0.05 versus control).

**ER-alpha but not ER-beta is suppressed in the middle-aged and old female hippocampus**

The hippocampus plays a critical role in learning and memory (37, 38). For that reason, neuroprotection of hippocampal cells in the face of challenges or insults, which often occur in aging brain (39), can result in improved cognitive function (40). For these reasons we have chosen to study the effects of age on ER-alpha and ER-beta mRNA expression in the hippocampus in addition to the cortex. As such, we have measured ER-alpha and ER-beta mRNA levels in the hippocampus of young, middle-aged, and old mice. Results (Fig. 2 top panel) show that relative to young, ER-alpha mRNA is 47% and 41% lower in the middle-aged and old mice, respectively, whereas ER-beta levels do not change with age, (p<0.05 versus control).

**A phytoestrogen rich diet increases hippocampal BDNF mRNA in the young mouse, does not affect levels in the middle-aged mouse, but decreases levels in the old mouse.**

To assess how phytoestrogens affect BDNF mRNA levels in the hippocampus as a function of age, we measured BDNF mRNA levels in the hippocampus of female mice that had initiated a phytoestrogen rich diet at a young, middle or old age. Results (Fig. 2 left side) show that relative to age-matched controls, a phytoestrogen rich diet caused a 22% reduction in BDNF mRNA relative to mice on a control (phytoestrogen free) diet, it did not
affect levels in the middle-aged mice, yet it caused a 21% increase in mRNA levels in the old mice, (p<0.05 versus control)

A phytoestrogen rich diet increases hippocampal BDNF protein in the young, middle-aged and old female

As was performed for the cortex, we also measured BDNF protein levels from the hippocampus of female mice that had initiated a phytoestrogen rich diet at a young, middle or old age. Results (Fig. 2 right side) show that compared to age-matched controls a phytoestrogen rich diet caused a 77%, 54%, and 57% increase in BDNF protein in the young, middle-aged, and old mice, respectively (p<0.05 versus control).

ER-alpha but not ER-beta decreases in the middle-aged and old male cortex

To assess how ER-alpha and ER-beta mRNA levels change in the male cortex, we measured cortical ER-alpha and ER-beta mRNA levels of young, middle-aged, and old male mice. Results (Fig. 3 top panel) show that relative to young mice, ER-alpha levels decrease by 28% and 53% in the middle-aged and old mice, respectively, while no age-related changes were observed for ER-beta mRNA levels (p<0.05 versus control).

A phytoestrogen rich diet increases cortical BDNF mRNA in the young and middle-aged but decreases levels in the old male mice

To assess how phytoestrogens affect BDNF mRNA in the male cortex, we measured BDNF mRNA levels from the cortex of male mice that had initiated a phytoestrogen rich diet at a young, middle, or old age. Results (Fig. 3 left side) show that relative to age-
matched controls, the phytoestrogen rich diet caused a 37% and 53% increase in mRNA levels in the young and middle-aged mice but a 38% decrease in the old male cortex (p<0.05 versus control).

**A phytoestrogen rich diet causes a decrease in BDNF mRNA levels in the old male cortex**

We measured BDNF protein levels from the cortex of male mice that had initiated a phytoestrogen rich diet at a young middle, or old age. Results (Fig. 3 right side) show that relative to age-matched controls, the phytoestrogen rich diet had no effect on BDNF mRNA levels in the young or middle-aged mice but caused a 38% decrease levels in the old mice (p<0.05 versus control).

**A phytoestrogen rich diet causes a decrease in cortical BDNF protein in the old male mouse**

To assess how phytoestrogens affect BDNF protein levels in the cortex of male mice, we measured BDNF from the cortex of male mice that had initiated a phytoestrogen rich diet at a young, middle, or old age. Results show (Fig. 3 right side) relative to age-matched controls, phytoestrogens had no effect on BDNF protein levels in the young or middle-aged mice, however they caused a 25% decrease in the old mice, (p<0.05 versus control).

**ER-alpha but not ER-beta increases in the hippocampus of old male mice**

To assess how ER-alpha and ER-beta mRNA levels change with age in the hippocampus of male mice, we measured ER-alpha and ER-beta mRNA levels from 9, 15,
and 27 month old male mice. Results (Fig. 4 top panel) show that there is a trend towards an age-related increase in ER-alpha with middle-aged mice having a 58% increase in ER-alpha mRNA and old mice having a 93% increase. Only the increase in the old mice was found to be statistically significant, (p<0.05 versus control). ER-beta levels remained unchanged with age.

**A phytoestrogen rich diet decreases hippocampal BDNF mRNA in middle-aged and old male mice**

To assess the effects that a phytoestrogen rich diet has on BDNF mRNA in the hippocampus of male mice, we measured BDNF mRNA levels from the hippocampus of male mice that had initiated a phytoestrogen rich diet at a young, middle, or old age.

Results (Fig. 4 left side) show that relative to age-matched controls, the phytoestrogen rich diet caused no changes in BDNF mRNA levels in young, but resulted in a 36% and 25% reduction in BDNF mRNA in the middle-aged, and old mice, respectively (p<0.05 versus control).

**A phytoestrogen rich diet increases hippocampal BDNF protein levels in young, middle-aged, and old male mice**

To assess the effects that a phytoestrogen rich diet has on BDNF protein levels in the male hippocampus, we measured hippocampal BDNF levels from male mice that had initiated a phytoestrogen rich diet at a young, middle or old age. Results (Fig. 4 right side) show that the phytoestrogens caused a 16%, 22%, and 27% increase in BDNF protein levels in young, middle-aged and old mice, respectively (p<0.05 versus control).
ER-beta but not ER-alpha decreases in the cortex of the old female mouse relative to the young female mouse

In the original analysis of our data (see above) there was no discernable relationship between expression of ER-alpha, ER-beta, or the combination thereof and BDNF mRNA and protein expression. We felt that perhaps the middle-aged group, because of potential variations in hormone levels and cyclicity, at least in the female mice, might be skewing the data. Therefore, we decided to reanalyze the data omitting the middle-aged group from our analysis and compare only young and old animals. In doing this analysis for ER-alpha and ER-beta in the female cortex, results show that ER-beta mRNA expression decreases by 24% in the cortex of the old female relative to the young (p<0.05 versus control). There was no change in ER-alpha expression in old compared to young (Fig. 5 top panel).

A phytoestrogen rich diet has no effect on cortical BDNF mRNA expression in young or old female mice

Having established that ER-beta and not ER-alpha changes with age in the female cortex, we then assessed the effect that a phytoestrogen rich diet has on BDNF mRNA expression in those same tissues. Results show that the phytoestrogen rich diet did not alter BDNF mRNA expression relative to the control (phytoestrogen free) fed group in young or old animals (Fig. 5 middle panel).
A phytoestrogen rich diet increases cortical BDNF protein in the young but not the old female mouse

The effect of phytoestrogens on cortical BDNF protein was assessed for females that had initiated a phytoestrogen rich diet at either a young or old age. Results show that a phytoestrogen rich diet doubled the amount of BDNF protein in the cortex of the young females (p<0.05 versus control diet) but had no effect on BDNF protein in the cortex of the old females (Fig. 5 bottom panel).

ER-alpha but not ER-beta decreases with age in the male cortex

Our analysis of ER expression in the young and old male cortex showed that ER-beta decreases by 53% in the old males relative to the young, (p<0.05 versus control); whereas no age-related change occurred for ER-alpha (Fig. 6 top panel).

A phytoestrogen rich diet increases cortical BDNF mRNA expression in young male mice but decreases expression in of old male mice

Quantification of BDNF mRNA expression from mice had initiated a phytoestrogen rich diet at 6 or 24 months shows that while a phytoestrogen rich diet increases cortical BDNF mRNA expression by 37% in young male mice, it caused a 38% reduction in expression in old male mice (p<0.05 versus control, Fig. 6 middle panel).
A phytoestrogen rich diet does not alter cortical BDNF protein levels in young male mice but causes a reduction in BDNF protein in the old male mice

The effect of phytoestrogens on cortical BDNF protein was assessed for males that had initiated a phytoestrogen rich diet at a young and old age. Results show that relative to control groups, phytoestrogens did not alter cortical BDNF protein levels in the young males but resulted in a 25% reduction of cortical BDNF protein in the old males (p<0.05 versus control, Fig. 6 bottom panel).

ER-alpha but not ER-beta decreases with age in the female hippocampus

Our analysis of ER mRNA expression in the young and old female hippocampus shows that ER-alpha decreases by 27% in the old female hippocampus relative to the young whereas ER-beta expression level did not change as a function of age (p<0.05 versus control, Fig. 7 top panel).

A phytoestrogen rich diet causes a reduction of hippocampal BDNF mRNA expression in the young female but an increase in the old female

Levels of BDNF mRNA from the hippocampus of female mice that had initiated a phytoestrogen rich diet at 6 or 24 months shows that relative to control, a phytoestrogen rich diet causes a 22% reduction of hippocampal BDNF mRNA expression in young females but a 20% increase in expression in the old females (p<0.05 versus control, Fig. 7 middle panel).
A phytoestrogen rich diet increases hippocampal BDNF protein in young and old female mice

The effect of phytoestrogens on hippocampal BDNF protein was assessed for females that had initiated a phytoestrogen rich diet at a young and old age. Results show that phytoestrogens caused a 77% and 57% increase in hippocampal BDNF protein in young and old female mice, respectively, (p<0.05 versus control, Fig. 7 bottom panel).

ER-alpha but not ER-beta is increased in the hippocampus of old male mice

Our analysis of ER mRNA expression in the young and old male hippocampus shows that ER-alpha mRNA expression increases by 93% in the old males relative to young whereas ER-beta levels remain relatively unchanged with age (p<0.05 versus control, Fig. 8 top panel).

A phytoestrogen rich diet causes a decrease in hippocampal BDNF mRNA expression in old but not young male mice

Quantification of BDNF mRNA expression from the hippocampus of male mice that had initiated a phytoestrogen rich diet at 6 or 24 months shows that relative to control, a phytoestrogen rich diet did not affect hippocampal BDNF mRNA expression in the young males but caused a 25% decrease in expression in the old males (p<0.05 versus control, Fig. 8 middle panel).
A phytoestrogen rich diet increases hippocampal BDNF protein in young and old male mice

The effect of phytoestrogens on hippocampal BDNF protein was assessed for males that had initiated a phytoestrogen rich diet at a young and old age. Results show that phytoestrogens caused a 15% and 27% increase in hippocampal BDNF protein in the young and old males respectively (p<0.05 versus control, Fig. 8 bottom panel).
ER-alpha mRNA in the female cortex

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ER-beta mRNA in the female cortex

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Effect of diet on BDNF mRNA in young female cortex

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Effect of diet on BDNF protein in young female cortex

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Effect of diet on BDNF mRNA in middle-aged female cortex

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Effect of diet on BDNF mRNA in old female cortex

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Effect of diet on BDNF protein in old female cortex

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Effect of diet on BDNF protein in young female cortex

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Effect of diet on BDNF protein in middle-aged female cortex

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Effect of diet on BDNF protein in old female cortex

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Figure 1. Top panel: Expression of ER-beta, but not ER-alpha mRNA in the female cortex increases in middle-aged mice. Female C57Bl6 mice that were all maintained on a phytoestrogen free diet were characterized for expression of ER-alpha and ER-beta mRNA. The cortex of these mice was harvested at the time of euthanasia. Mice belonged to one of three age groups: 9 months, 15 months, or 27 months. The cortical tissue was lysed from which RNA was isolated. RNA was subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure ER-alpha and ER-beta mRNA levels. Levels obtained from the young mice were set to one and results are shown as a fold change relative to that group. Results show that cortical ER-alpha mRNA expression remained relatively constant with age, but ER-beta mRNA levels increased by 87% (* denotes a statistically significant difference as compared to young; p<0.05 versus control). Left side: A phytoestrogen rich diet suppresses BDNF mRNA levels in the cortex of middle-aged female mice. At 6 months of age, 12 months of age, or 24 months of age, C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed the diet for a period of 13 weeks, during part of which the animals underwent behavioral testing (data not reported here). After the 13 weeks, the animals were euthanized. After euthanasia, the cortex was harvested from the mice and the tissue was lysed. RNA was isolated from the lysate and subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels of BDNF. Data are expressed as a function of diet relative to the control (phytoestrogen free) group which has been set to 1. Results show that relative to age-matched controls, the phytoestrogen rich diet only affected the BDNF mRNA levels in the middle-aged group, causing a 21% decrease, (* denotes statistical significance from the phyto-free group; p<0.05 versus control). Right
side: A phytoestrogen rich diet increases BDNF protein in the young and middle-aged female cortex. Tissue from the mice described above was also used to measure BDNF protein. The cortical tissue was lysed and an ELISA was performed to quantify BDNF protein levels. Data is expressed in pg/ml. Results show that the phytoestrogen rich diet caused a 100% and 150% increase in the young and middle-aged female cortex but had no effect in the old animals, (* denotes statistical significance from the phyto-free group; p<0.05 versus control).
Figure 2. Top panel: ER-alpha, but not ER-beta mRNA levels decrease in the middle-aged and old female cortex. C57Bl6 mice which were all maintained on a phytoestrogen free diet were characterized for expression of ER-alpha and ER-beta. The hippocampus of these mice was harvested at the time of euthanasia. Mice belonged to one of three age groups: 9 months, 15 months, or 27 months. The hippocampal tissue was lysed and RNA was isolated from the lysate. RNA was subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure ER-alpha and ER-beta mRNA levels. Levels obtained from the young mice were set to one and results are shown as a fold change relative to that group. Results show that relative to young, ER-alpha mRNA is 47% and 41% lower in the middle-aged and old mice, respectively, whereas ER-beta levels do not change with age, (* denotes a statistically significant difference as compared to young; p<0.05 versus control). Left side: A phytoestrogen rich diet increases hippocampal BDNF mRNA in the young and middle-aged female. At 6 months of age, 12 months of age, or 24 months of age, C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed the diet for a period of 13 weeks, during part of which the animals underwent behavioral testing (data not reported here). After the 13 weeks, the animals were euthanized. After euthanasia, the hippocampus was harvested from the mice and the tissue was lysed. RNA was isolated from the lysate and subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels of BDNF. Data are expressed as a function of diet relative to the control (phytoestrogen free) group which has been set to 1. Results show that relative to age-matched controls, a phytoestrogen rich diet caused a 22% reduction in BDNF mRNA relative to mice on a control (phytoestrogen free) diet, it did not affect levels in the middle-
aged mice, yet it caused a 21% increase in mRNA levels in the old mice (* denotes statistical significance from the phyto-free group; p<0.05 versus control). Right side: A phytoestrogen rich diet causes an increase in BDNF protein in the female hippocampus. Tissue from the mice described above was also used to measure BDNF protein. The cortical tissue was lysed and an ELISA was performed to quantify BDNF protein levels. Data is expressed in pg/ml. Results show that compared to age-matched controls a phytoestrogen rich diet caused a 77%, 54%, and 57% increase in BDNF protein in the young, middle-aged, and old mice, respectively (* denotes a statistical significance from the phyto-free group; p<0.05 versus control).
Effect of diet on BDNF mRNA in young male cortex

Effect of diet on BDNF mRNA in middle-aged male cortex

Effect of diet on BDNF mRNA in old male cortex

Effect of diet on BDNF protein in young male cortex

Effect of diet on BDNF protein in middle-aged male cortex

Effect of diet on BDNF protein in old male cortex
Figure 3. Top panel: ER-alpha, but not ER-beta decreases in the middle-aged and old male cortex. Male C57Bl6 mice that were all maintained on a phytoestrogen free diet were characterized for expression of ER-alpha and ER-beta mRNA. The cortex of these mice was harvested at the time of euthanasia. Mice belonged to one of three age groups: 9 months, 15 months, or 27 months. The cortical tissue was lysed from which RNA was isolated. RNA was subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure ER-alpha and ER-beta mRNA levels. Levels obtained from the young mice were set to one and results are shown as a fold change relative to that group. Results show that relative to young mice, ER-alpha levels decrease by 28% and 53% in the middle-aged and old mice, respectively, while no age-related changes were observed for ER-beta mRNA levels (* denotes a statistical difference from young; p<0.05 versus control). Left side: A phytoestrogen rich diet increases cortical BDNF mRNA in the young and middle-aged mice but decrease it in the old mice. At 6 months of age, 12 months of age, or 24 months of age, C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed the diet for a period of 13 weeks, during part of which the animals underwent behavioral testing (data not reported here). After the 13 weeks, the animals were euthanized. After euthanasia, the cortex was harvested from the mice and the tissue was lysed. RNA was isolated from the lysate and subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels of BDNF. Data are expressed as a function of diet relative to the control (phytoestrogen free) group which has been set to 1. Results show that relative to age-matched controls, the phytoestrogen rich diet caused a 37% and 53% increase in mRNA levels in the young and middle-aged mice but a 38% decrease in the old male cortex (* denotes a statistical
difference from the phyto-free group; p<0.05 versus control). Right side: A phytoestrogen rich diet decreases BDNF in the cortex of old male mice. Tissue from the mice described above was also used to measure BDNF protein. The cortical tissue was lysed and an ELISA was performed to quantify BDNF protein levels. Data is expressed in pg/ml. Results show that relative to age-matched controls, the phytoestrogen rich diet had no effect on BDNF mRNA levels in the young or middle-aged mice but caused a 38% decrease levels in the old mice (* denotes a statistical difference from the phyto-free group; p<0.05 versus control).
ER-alpha mRNA in the male hippocampus

**Effect of diet on BDNF mRNA in young male hippocampus**

**Effect of diet on BDNF protein in young male hippocampus**

**Effect of diet on BDNF mRNA in middle-aged male hippocampus**

**Effect of diet on BDNF protein in middle-aged male hippocampus**

**Effect of diet on BDNF mRNA in old male hippocampus**

**Effect of diet on BDNF protein in old male hippocampus**
Figure 4. Top panel: ER-alpha, but not ER-beta increases in the hippocampus of the old male mouse. Male C57Bl6 mice that were all maintained on a phytoestrogen free diet were characterized for expression of ER-alpha and ER-beta mRNA. The hippocampus of these mice was harvested at the time of euthanasia. Mice belonged to one of three age groups: 9 months, 15 months, or 27 months. The hippocampal tissue was lysed from which RNA was isolated. RNA was subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure ER-alpha and ER-beta mRNA levels. Levels obtained from the young mice were set to one and results are shown as a fold change relative to that group. Results show that there is a trend towards an age-related increase in ER-alpha with middle-aged mice having a 58% increase in ER-alpha mRNA and old mice having a 93% increase. Only the increase in the old mice was found to be statistically significant (* denotes a statistical difference from young; p<0.05 versus control). ER-beta levels remained unchanged with age. Left side: A phytoestrogen rich diet causes a reduction in BDNF mRNA levels in the middle-aged and old male mice. At 6 months of age, 12 months of age, or 24 months of age, C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed the diet for a period of 13 weeks, during part of which the animals underwent behavioral testing (data not reported here). After the 13 weeks, the animals were euthanized. After euthanasia, the cortex was harvested from the mice and the tissue was lysed. RNA was isolated from the lysate and subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels of BDNF. Data are expressed as a function of diet relative to the control (phytoestrogen free) group which has been set to 1. Results show that relative to age-matched controls, the phytoestrogen rich diet caused no changes in BDNF mRNA levels
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Effect of diet on BDNF in young female cortex

- Phyto-free
- Phyto-rich

Effect of diet on BDNF in old female cortex

- Phyto-free
- Phyto-rich

Effect of diet on BDNF protein in young female cortex

- Phyto-free
- Phyto-rich

Effect of diet on BDNF protein in old female cortex

- Phyto-free
- Phyto-rich

Effect of Age on ER-alpha in female cortex

- Young
- Old

Effect of Age on ER-beta in female cortex

- Young
- Old

Effect of Age on ER-alpha in female cortex

- Young
- Old

Effect of Age on ER-beta in female cortex

- Young
- Old

Effect of Age on ER-alpha in female cortex

- Young
- Old

Effect of Age on ER-beta in female cortex

- Young
- Old
Figure 5. Top panel: ER-beta but not ER-alpha decreases in the cortex of the old female mouse relative to the young female mouse. C57Bl6 mice which were all maintained on a phytoestrogen free diet were characterized for expression of ER-alpha and ER-beta. The cortex of the female mice was harvested at the time of euthanasia. Here we are reporting results from the two age groups 6 months of age and 24 months of age. The cortical tissue was lysed from which RNA was isolated. RNA was subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure ER-alpha and ER-beta mRNA levels. Levels obtained from the young mice were set to one and results are shown as a fold change relative to that group. Results show that relative to the young mice, ER-alpha does not change with age however ER-beta expression is decreased by 24% in the old mice relative to the young mice. (* denotes a statistically significant difference as compared to young; p<0.05 versus control). Middle Panel: A phytoestrogen rich diet has no effect on BDNF mRNA expression in the cortex of young or old female mice. At 6 months of age or 24 months of age C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed that diet for 13 weeks. After euthanasia, the cortex was harvested from the female mice and the tissue was lysed. RNA was isolated from the lysate and subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels of BDNF. Data are expressed as a function of diet relative to the control (phytoestrogen free) group which has been set to 1. Results show that phytoestrogens did not affect cortical BDNF mRNA levels in young or old females. Bottom panel: A phytoestrogen rich diet increases BDNF protein in the cortex of the young but not the old female mouse. At 6 months of age or 24 months of age C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen
rich diet. Mice consumed that diet for 13 weeks. After euthanasia, the cortex was harvested from the female mice and lysed. An ELISA was performed using the lysate to quantify BDNF protein levels. Data is expressed in pg/ml. Results show that phytoestrogens caused a doubling of cortical BDNF protein in the young females but had no effect on cortical BDNF protein levels in the old females. (* denotes a statistically significant difference as compared to the phytoestrogen free group; p<0.05 versus control)
Effect of Age on ER-alpha in female hippocampus

**Fold change (compared to young)**

- **Young**: 1.0
- **Old**: 0.5

*Significant difference

---

Effect of Age on ER-beta female hippocampus

**Fold change (compared to young)**

- **Young**: 1.0
- **Old**: 1.0

**Effect of diet on BDNF protein in young female hippocampus**

**BDNF (pg/ml)**

- **Phyto-free**: 15
- **Phyto-rich**: 25

*Significant difference

---

**Effect of diet on BDNF protein in old female hippocampus**

**BDNF (pg/ml)**

- **Phyto-free**: 15
- **Phyto-rich**: 25

*Significant difference

---

Effect of diet on BDNF mRNA in young female hippocampus

**Fold change (compared to phyto-free diet)**

- **Phyto-free**: 1.0
- **Phyto-rich**: 0.75

*Significant difference

---

Effect of diet on BDNF mRNA in old female hippocampus

**Fold change (compared to phyto-free diet)**

- **Phyto-free**: 1.0
- **Phyto-rich**: 1.25

*Significant difference

---

Effect of diet on BDNF protein in young female hippocampus

**BDNF (pg/ml)**

- **Phyto-free**: 15
- **Phyto-rich**: 25

*Significant difference

---

Effect of diet on BDNF protein in old female hippocampus

**BDNF (pg/ml)**

- **Phyto-free**: 15
- **Phyto-rich**: 25

*Significant difference
Figure 6. Top panel: ER-alpha but not ER-beta decreases with age in the female hippocampus. C57Bl6 mice which were all maintained on a phytoestrogen free diet were characterized for expression of ER-alpha and ER-beta. The hippocampus of the female mice was harvested at the time of euthanasia. Here we are reporting results from the two age groups 6 months of age and 24 months of age. The hippocampal tissue was lysed and RNA was isolated from the lysate. RNA was subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels for ER-alpha and ER-beta. Levels obtained from the young mice were set to one and results are shown as a fold change relative to that group. Results show that ER-alpha mRNA expression decreases by 27% as a function of age whereas ER-beta expression does not change. (* denotes a statistically significant difference as compared to young; p<0.05 versus control). Middle panel: A phytoestrogen rich diet causes a reduction of BDNF mRNA expression in the young female hippocampus but an increase in the old female hippocampus. At 6 months of age or 24 months of age C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed that diet for 13 weeks. After euthanasia, the hippocampus was harvested from the female mice and lysed. RNA was isolated from the tissue lysate and subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels of BDNF. Data are expressed as a function of diet relative to the control (phytoestrogen free) group which has been set to 1. Results show that phytoestrogens differentially regulated BDNF mRNA expression causing a 22% reduction in the young females and a 20% increase in the old females. (* denotes a statistically significant difference as compared to the phytoestrogen free group; p<0.05 versus control). Bottom panel: A phytoestrogen rich diet increases BDNF protein in the
hippocampus of young and old female mice. At 6 months of age or 24 months of age C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed that diet for 13 weeks. After euthanasia, the hippocampus was harvested from the female mice and the tissue was lysed. An ELISA was performed using the lysate to quantify BDNF protein levels. Data is expressed in pg/ml. Results show that phytoestrogens increased hippocampal BDNF protein levels by 77% and 57% in young and old female mice, respectively. (* denotes a statistically significant difference as compared to the phytoestrogen free group; p<0.05 versus control)
Effect of Age on ER-alpha in male cortex

Effect of Age on ER-beta in male cortex

Effect of diet on BDNF in young male cortex

Effect of diet on BDNF in old male cortex

Effect of diet on BDNF protein in young male cortex

Effect of diet on BDNF protein in old male cortex
Figure 7. Top panel: ER-alpha but not ER-beta decreases with age in the male cortex. C57Bl6 mice which were all maintained on a phytoestrogen free diet were characterized for expression of ER-alpha and ER-beta. The cortex of the male mice was harvested at the time of euthanasia. Here we are reporting results from the two age groups 6 months of age and 24 months of age. The cortical tissue was lysed and RNA was isolated from the lysate. RNA was subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels for ER-alpha and ER-beta. Levels obtained from the young mice were set to one and results are shown as a fold change relative to that group. Results show that while ER-beta mRNA expression decreased by 53% in the old mouse relative to the young, ER-alpha expression does not change from young to old. (* denotes a statistically significant difference as compared to the young group; p<0.05 versus control). Middle panel: A phytoestrogen rich diet increases BDNF mRNA expression in the cortex of young male mice but decreases expression in the cortex of old male mice. At 6 months of age or 24 months of age C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed that diet for 13 weeks. After euthanasia, the cortex was harvested from the male mice and the tissue was lysed. RNA was isolated from the tissue and subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels of BDNF. Data are expressed as a function of diet relative to the control (phytoestrogen free) group which has been set to 1. Results show that while phytoestrogens caused a 37% increase in cortical BDNF mRNA expression in the young mice relative to control, they caused a 38% reduction in expression in the old mice relative to control. (* denotes a statistically significant difference as compared to the phytoestrogen free group; p<0.05 versus control). Bottom panel: A
phytoestrogen rich diet does not alter BDNF protein levels in the cortex of young male mice but causes a reduction in BDNF protein in the old male mice. At 6 months of age or 24 months of age C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed that diet for 13 weeks. After euthanasia, the cortex was harvested from the male mice and lysed. An ELISA was performed using the lysate to quantify BDNF protein levels. Data is expressed in pg/ml. Results show that phytoestrogens had no effect on cortical BDNF protein levels in the young males yet they caused a 25% reduction in BDNF protein levels in the old males. (* denotes a statistically significant difference as compared to the phytoestrogen free group; p<0.05 versus control)
Effect of diet on BDNF protein in young male hippocampus

![Diagram showing effect of diet on BDNF protein in young male hippocampus](image)

Effect of diet on BDNF protein in old male hippocampus

![Diagram showing effect of diet on BDNF protein in old male hippocampus](image)

Effect of diet on BDNF mRNA in young male hippocampus

![Diagram showing effect of diet on BDNF mRNA in young male hippocampus](image)

Effect of diet on BDNF mRNA in old male hippocampus

![Diagram showing effect of diet on BDNF mRNA in old male hippocampus](image)

Effect of Age on ER-alpha in male hippocampus

![Diagram showing effect of Age on ER-alpha in male hippocampus](image)

Effect of Age on ER-beta in male hippocampus

![Diagram showing effect of Age on ER-beta in male hippocampus](image)
Figure 8. Top panel: ER-alpha but not ER-beta is increased in the hippocampus of old male mice. C57Bl6 mice which were all maintained on a phytoestrogen free diet were characterized for expression of ER-alpha and ER-beta. The hippocampus of the male mice was harvested at the time of euthanasia. Here we are reporting results from the two age groups 6 months of age and 24 months of age. The hippocampal tissue was lysed and RNA was isolated from the lysate. RNA was subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels for ER-alpha and ER-beta. Levels obtained from the young mice were set to one and results are shown as a fold change relative to that group. Results show that ER-alpha increases by 93% in the old relative to the young but ER-beta does not change significantly with age. (* denotes a statistically significant difference as compared to young, p<0.05 versus control). Middle panel: A phytoestrogen rich diet causes a decrease in BDNF mRNA expression in the hippocampus of old but not young male mice. At 6 months of age or 24 months of age C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed that diet for 13 weeks. After euthanasia, the hippocampus was harvested from the male mice. RNA was isolated from the tissue and subsequently reverse transcribed into cDNA which was then used for real time RT-PCR to measure mRNA levels of BDNF. Data are expressed as a function of diet relative to the control (phytoestrogen free) group which has been set to 1. Results show that phytoestrogens have no effect on hippocampal BDNF mRNA expression in the young males but decreased expression by 25% in the old males. (* denotes a statistically significant difference as compared to the phytoestrogen free group, p<0.05 versus control). Bottom panel: A phytoestrogen rich diet increases BDNF protein in the hippocampus of young and
old male mice. At 6 months of age or 24 months of age C57Bl6 mice were either maintained on the phytoestrogen free diet or switched to a phytoestrogen rich diet. Mice consumed that diet for 13 weeks. After euthanasia, the hippocampus was harvested from the female mice and the tissue was lysed. An ELISA was performed using the lysate to quantify BDNF protein levels. Data is expressed in pg/ml. Results show that phytoestrogens increased hippocampal BDNF protein levels by 15% and 27% in young and old male mice respectively. (*) denotes a statistically significant difference as compared to the phytoestrogen free group, p<0.05 versus control).
### Females

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**BDNF mRNA**

- No change
- Decrease
- Decrease
- No change
- No change
- Increase

**BDNF protein**

- Increase
- Increase
- Increase
- Increase
- No change
- Increase
Table 1. Summary of phytoestrogen rich diet induced changes in BDNF expression in females. Table 1 summarizes the changes in BDNF mRNA and protein observed in the cortex and hippocampus of female mice that had consumed a phytoestrogen rich diet.
### Males

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<table>
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Table 2. Summary of phytoestrogen rich diet induced changes in BDNF expression in males.

Table 1 summarizes the changes in BDNF mRNA and protein observed in the cortex and hippocampus of male mice that had consumed a phytoestrogen rich diet.
DISCUSSION

Soy, because of its high plant protein content and many health benefits (41-46) could be considered a superfood. Adding to its allure is the potential of the phytoestrogens found in soy to improve brain function. Because phytoestrogens’ effects on the brain are less understood than those in the periphery, clinical studies have aimed at documenting the cognitive enhancing effects of phytoestrogens. Results of these studies have resulted in more questions than answers. Overall, phytoestrogens improved cognition in some studies (6-8) and worsened cognitive function in others (9, 10) leaving scientists to question where the discrepancy lies. Our previous work with the phytoestrogen genistein led us to hypothesize that ERs are essential for it to elicit neuroprotective effects. For that reason, in this study we have evaluated ER-alpha and ER-beta mRNA expression in the cortex and hippocampus of male and female mice and how that expression changes as a function of age. We hypothesize that if age causes suppression in ER expression, phytoestrogens may lose their neuroprotective properties. Here we used BDNF as a surrogate marker for neuroprotection. We chose BDNF as our surrogate marker because it activates pro-survival signaling pathways (47, 48) and has been implicated in improved cognitive performance (49). In addition, it has been shown to be essential for hormone induced neuroprotection in studies done by our lab (34). In these studies we have used real time RT-PCR and a sandwich ELISA method to study the effect that a phytoestrogen rich diet has on BDNF mRNA and protein expression in the cortex and hippocampus of male and female mice when the diet is initiated at 6 months, 12 months, or 24 months to represent young, middle-aged, and old mice, respectively.
With respect to the effect that age has on ER mRNA, the data show that ER-beta remains unchanged with age in the cortex and hippocampus of male mice and in the hippocampus of the female mice. In our initial analysis of the data, the only observed age-related change in ER-beta mRNA occurred in the cortex of the middle-aged female mice (Fig. 1 top, right). We speculate that this may be a transient compensatory response to the decrease in endogenous estrogen which occurs in middle-aged female mice (50). Levels of ER-alpha, on the other hand were quite sensitive to the age of the animal. Although age does not affect ER-alpha expression in the female cortex (Fig. 1, top left), there is an age-related decrease in the hippocampus of the female mice (Fig. 2, top left). The male cortex responded differently than the female cortex in that there was an age-related decrease in ER-alpha (Fig. 3, top left). The hippocampus of the male mice showed and increase in ER-alpha in the old mice only (Fig. 4, left). Overall, levels of ER-beta expression did not change with age with the exception of the middle-aged females (Fig. 1, right). ER-alpha levels are more subject to change with age, increasing with age in the male hippocampus and decreasing with age in both the male cortex and the female hippocampus.

Given that we ultimately wanted to determine if we could identify a relationship between ER expression and phytoestrogen neuroprotective effects, as indexed by BDNF as a surrogate marker of neuroprotection, our next objective was to identify how a diet rich in phytoestrogens affected BDNF mRNA and protein level. Male and female mice were initiated on a phytoestrogen rich diet at either 6, 12 or 24 months of age and maintained on that diet for a period of 13 weeks. During part of the 13 week period, animals underwent behavioral testing (data not presented here). Data obtained from these mice were compared against age-matched control mice that remained on a comparable but
phytoestrogen free diet. Regarding BDNF mRNA expression, data show that in the female cortex, phytoestrogens only had an effect in the middle-aged group causing a decrease in expression relative to control (Fig. 1, left). In the female hippocampus, the phytoestrogen rich diet decreased BDNF mRNA in the young mouse relative to control, did not alter mRNA levels in the middle-aged group but resulted in an increase in BDNF mRNA expression in the old mice (Fig. 2, left). In the male cortex, the phytoestrogen rich diet increased BDNF in the young and middle-aged but not the old mice (Fig. 3, left), however it decreased expression in the hippocampus of the middle-aged and old mice not affecting the young (Fig. 4, left). This data indicates that the effect that phytoestrogens have on BDNF mRNA expression in the cortex and hippocampus is dependent on the age at which the diet was initiated. The sex of the animal is also a factor.

In order to use BDNF as our surrogate marker of neuroprotection it was necessary to assess how phytoestrogens affect the protein levels of BDNF. Our observations are that the protein levels are not always affected the same way the mRNA levels are, hinting at differential regulation of transcriptional and post-transcriptional processes within the cells. In the female cortex, the phytoestrogen rich diet caused an increase in BDNF protein in the young and middle-aged mice but not in the old mice (Fig. 1, right). The males, on the other hand, showed no changes in BDNF protein in the cortex of young or middle-aged mice but did show a decrease in cortical BDNF protein in the old animal (Fig. 3, right). The phytoestrogen rich diet caused an increase in hippocampal BDNF protein level in both males and females at all of the ages tested (Fig. 2, right and Fig. 4, right). Changes in BDNF mRNA and protein levels that resulted from the phytoestrogen rich diet have been summarized in Table 1 (female data) and Table 2 (male data). We have used this collective
BDNF data to make predictions regarding whether or not phytoestrogens will be beneficial or not in the male and female brain. We make the following predictions based on the fact that BDNF has been linked to neuroprotection and improved cognition (16, 17, 51). If one assumes that higher BDNF protein levels would result in improved memory and cognition, our data suggests that females, particularly young and middle-aged, would respond favorably to higher amounts of dietary phytoestrogens. Males on the other hand may not respond favorably or may even have a negative response based on the suppression that phytoestrogens exerted on BDNF protein in the cortex of the older males. The fact that phytoestrogens increased BDNF in the male hippocampus but had no effect or decreased BDNF in the male cortex adds a level of complexity in deciphering an overall effect of phytoestrogens with respect to cognitive function. There may in fact be a region specific functional outcome in response to phytoestrogens.

It is worth pointing out that our assessment of BDNF protein did not discriminate between the pro- and mature forms of BDNF. Given the potentially different role of pro-versus mature BDNF in regulating cell viability, additional analysis may be required to gain insight into the significance of the changes in BDNF protein levels measured.

In clinical studies where memory and cognition are evaluated, outcomes are determined by measuring certain end points based on particular memory related tasks. It may be that the outcome of the study depends on which brain region dominates control over the particular task or tasks being measured in that study. For example, hippocampal function has been shown to be essential in episodic and declarative memory (37, 38) but not for procedural memory or priming which depend on cortical function (52). Therefore, if the endpoint being measured is driven by the hippocampus, results will suggest that
phytoestrogens improve memory given that the data presented here demonstrates that phytoestrogens upregulate BDNF in the hippocampus. If, on the other hand, the endpoint being measured is driven by cortical function, the results might suggest that phytoestrogens do not improve or perhaps even worsen cognitive function in males given that the data presented here demonstrate that phytoestrogens do not increase BDNF in the male cortex and even decrease it in the older male mouse.

In our initial analysis of relative ER abundance and the effect that phytoestrogens have in the cortex and hippocampus, there was no discernable relationship to be made. We felt that perhaps, the middle-aged group could be skewing results, in part because of the variation in hormone levels and/or cyclicity in the female mice. We therefore did a secondary analysis comparing only the young and old animals. In this analysis we determined that ER-beta but not ER-alpha decreases with age in the female cortex (Fig. 5, top panel). Associated with that is the lack of effect of phytoestrogens at either age on BDNF transcription (Fig. 5, middle panel) but an increase in protein level (Fig. 5 bottom panel), presumably a result of increased translation in the young but not old females. Because ER-beta decreased in the same group of animals where we failed to see a translational effect of phytoestrogens, we speculated that ER-beta is the receptor that is driving translation in response to phytoestrogens. Because phytoestrogens are not changing transcriptional processes in the young or old female cortex, we speculate the transcriptional processes in this brain region may be driven by ER-beta at an ERE site. This interpretation is based on the work of Paech et. al., who showed that partial ER agonists which bind to ER-beta have no transcriptional effects when they act at ERE sites (53). In this scenario, we interpret phytoestrogens as partial agonists to the ER. The data obtained
from the female hippocampus shows that ER-alpha but not ER-beta decreases with age (Fig. 6, top panel) and that phytoestrogens decrease BDNF transcription in the young females but increase it in the old females (Fig. 6, middle panel). Phytoestrogens increased BDNF protein at both ages in this group (Fig. 6, bottom panel). This data is consistent with our theory that ER-beta is the receptor responsible for driving translation as it is unchanged between young and old, and the protein levels increase in both young and old animals. Like the cortex, we believe that transcriptional regulation is occurring through ER-beta in the hippocampus as well, however we speculate that in this region transcription is driven by ER-beta at an AP1 site. It has been shown that when partial agonists bind to ER-beta and act at an AP1 site, they increase transcription, albeit with less efficiency than a full agonist (53). In the young mice, where estradiol levels are presumed high, phytoestrogens can act as a partial agonist, competing away the effects of estradiol and thereby decreasing the transcriptional effects being driven by estradiol. However, in the older females, where estradiol levels presumed to be lower and competition for ER-beta less likely to occur, phytoestrogens can act as a partial agonist to increase transcription.

The data obtained from males in this study do not fit into our proposed model; however we attribute this to fundamental differences between the males and females which make predicting a model for the effects of phytoestrogens in males considerably more difficult. Such differences include the aromatization of testosterone to estradiol, the relatively higher abundance of dihydrotosterone (DHT) in males, which can be metabolized into 5alpha-androstane-3beta,17-beta-diol (3beta-diol), an ER-beta selective agonist (54-56), and the presence of
androgens and their receptors. Further characterization of the male brain, is needed to explain the mechanism by which phytoestrogens affect BDNF in the brain.

Here, we have established that phytoestrogens alter BDNF levels and that the effects are dependent upon age, sex, and brain region. We have put forth a theory on the way in which we think phytoestrogens are working in the female brain as supported by our data. The data presented here is useful when considering whether to initiate a soy diet or phytoestrogen treatment, or even more so as researchers design future human studies investigating the effects of soy and phytoestrogens on the brain.
REFERENCES


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<td>20.</td>
<td><strong>Sihil M, Bonnichon F, Rage F, Tapia-Arancibia L</strong> 2005 Age-related changes in brain-derived neurotrophic factor and tyrosine kinase receptor isoforms in the hippocampus and hypothalamus in male rats. <em>Neuroscience</em> 132:613-24</td>
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Chapter IV

DISCUSSION

Current research has demonstrated that when determining whether phytoestrogens improve brain function, the answer is more than a simple yes or no. It was this complexity which prompted us to investigate the circumstances under which the brain may benefit from phytoestrogens. It was our initial hypotheses that phytoestrogens are in fact neuroprotective. As a result of our initial studies, we added to our hypothesis the premise that the protective effects of phytoestrogens on the brain are age and sex dependent and that certain key players involved, which may differ as a function of age and sex, likely predict the effects that phytoestrogens have on the brain. The results presented herein support the hypothesis and shed light on the factors necessary for phytoestrogens to have a beneficial effect on the brain. Such information is relevant and important due to the fact that phytoestrogens, which can be purchased over the counter, are classified as an herbal supplement and therefore are not regulated by the Food and Drug
Administration. This means that no requirement exists to check for safety and short or long term effects. Many people do consume high amounts of phytoestrogens either as a natural part of their diet or through supplementation in hopes of receiving some of the other cited health benefits such as lowering the risk of certain cancers, lowering both LDL and total cholesterol levels, and relieving menopausal hot flashes (1-7). With so many people consuming soy or phytoestrogens regularly, it is critical to determine what the effects might be on the brain.

Out of a need to establish whether phytoestrogens have a positive or negative effect on cognition, clinical studies have been done in which subjects consumed specified amounts of phytoestrogens and are then scored on different memory related tasks. The results of such studies however have not provided a straightforward answer as to whether phytoestrogens are improving cognitive function or not. The reason for this is that some studies have reported improved memory with higher phytoestrogen intake (8-10) whereas other studies have reported a worsening effect of soy and phytoestrogens on memory and cognition (11, 12). This discrepancy in the outcomes of clinical studies is the basis for a need to further investigate how phytoestrogens function in the brain and what defines the circumstances under which they may be beneficial as opposed to detrimental to cognitive function.

In our initial studies we used cellular and ex vivo tissue culture models to assess whether genistein, which is the most abundant phytoestrogen in soy, is able to protect brain cells from cell death in the presence of an age-related insult. Our
results indicated that in fact genistein was able to protect in some instances but not in all the models in which we studied. For example, genistein protected both the cerebral cortical explants and the cortical HCN-1A cells from a glutamate-induced cell death but had no effect on the hippocampal derived HT22 cell line treated with either glutamate or H$_2$O$_2$. Based on an analysis of ER expression profiles in these three model systems, we came to the conclusion that the difference in genistein’s effects is a function of the ER status in the models used. Genistein is known to bind to and activate the ER and it does so with a 20-fold higher affinity for ER-beta than ER-alpha (13). For this reason, we believe ER-beta is a critical player in genistein-induced neuroprotection. Our laboratory has shown that while the explants express both ER-alpha and ER-beta, the HT22 cells only expressed ER-alpha. It is perhaps the lack of ER-beta in the HT22 cells that prevents genistein from being able to protect those cells against insult. It is unknown whether the HCN-1A cells express ER-alpha or ER-beta but those cells have been shown to respond to estradiol in a concentration dependent manner (14) and are protected by other phytoestrogens such as red clover (15). To further support the notion that the ER is required for genistein to exert neuroprotective effects, our studies show that the neuroprotective effect of genistein against glutamate in the explant model was abolished when the ER was blocked by the receptor antagonist ICI 182,780.

If, in fact, the ER mediates genistein’s protective effects on the brain, it is foreseeable that genistein’s effects may differ in males and females and may also depend on age given that levels of ER differ in males and females and with age (16, 17). To evaluate how the expression of ERs and effects of phytoestrogens differ with
sex and age, we have measured ER-alpha and ER-beta mRNA levels as a function of age in the cortex and hippocampus of C57Bl/6 mice and tested a model in which male and female mice were initiated on a diet rich in phytoestrogens at 6 months, 12 months, or 24 months of age as a representation of young, middle-aged and old, respectively. The mice consumed this diet for 5 weeks, after which they were euthanized and the brain tissue was harvested for biochemical/molecular analysis. Our results show that ER-alpha and ER-beta mRNA is present in males and females at all age groups. In females, ER-alpha expression decreased with age in the hippocampus but not in the cortex. ER-beta expression in the female remains mostly unchanged with an exception of a middle-aged spike in the cortex. Because this is the only observed change in ER-beta mRNA expression in the animals and it coincides with the stage in life when a female mouse would experience a mid-life decrease in endogenous estrogens (18), we speculate that this observation might be a transient compensatory response to such a decline in estrogen. While expression levels of ER-beta do not change in the males within either brain region, ER-alpha decreases with age in the cortex and increases with age in the hippocampus. Taken together, this data indicates the presence of ER-alpha and ER-beta mRNA in both males and females and shows the existence of age related changes in expression which could potentially affect how phytoestrogens affect the brain.

A major objective of this study was to assess whether a correlation could be made between the ER expression profile and how that affects the neuroprotective properties of phytoestrogens. As a way to evaluate how phytoestrogens protect the brain, we have chosen to measure BDNF as a surrogate marker of neuroprotection.
Genistein has been shown to alter BDNF mRNA in the rat cortex (19, 20). BDNF is a neurotrophin which can protect brain cells through activation of pro-survival signaling pathways such as the MAPK and PI3K pathways (21-23) and has been shown to improve reference memory (24). Our lab has already shown that BDNF is required for hormone-induced neuroprotection against an excitotoxic glutamate insult in the explant model (25) and thus, it is quite feasible that BDNF plays a role in genistein’s effects as well. It is for this reason that we measured BDNF mRNA levels in the cortex and the hippocampus of the mice used in this study. Our observations are that phytoestrogens alter BDNF mRNA in an age and sex dependent manner increasing expression in the young and middle-aged male cortex and decreasing it in the old male cortex and the middle-aged female cortex. In the hippocampus phytoestrogens decreased BDNF mRNA expression in the middle-aged and old males, however in the females, phytoestrogens decreased expression in the young, had no effect in the middle-aged and increased expression in the old mice.

To be able to predict a functional significance of phytoestrogens’ effects on the brain we also measured BDNF protein levels and found that they do not always change in the same direction as the mRNA levels indicating differential regulation of transcription and post-transcriptional processes. Results showed that phytoestrogens increased BDNF protein in the hippocampus at all age groups in both the male and female mice. Such an increase could result in improved brain function, especially with respect to memory. The cortex however had a more sex dependent response to phytoestrogens. The phytoestrogen rich diet resulted in an increase in BDNF in the cortex of the young and middle-aged females. No change
was observed in the old females. In the male mice, phytoestrogens had no effect on BDNF in the young and middle-aged mice, but did result in a decrease in the old mice. The results suggest that phytoestrogens have differing effects in the two brain regions most involved in learning and memory with respect to BDNF. Within the cortex a sex difference is apparent.

A region specific effect of phytoestrogens on BDNF protein potentially explains why the clinical studies are reporting varying results. When such clinical studies are performed, effects of phytoestrogens on memory are measured by a participant’s performance on certain memory related tasks. Different tasks measure different types of memory. Some memory such as episodic and declarative memory have been shown to require intact hippocampal function (26, 27) while other forms of memory such as procedural memory and priming do not require the hippocampus but depend more on cortical function (28). Because the cortex and hippocampus respond differently to phytoestrogens in terms of BDNF levels, whichever region governs the type of memory measured by a particular task in a study may determine whether or not that study shows improvement, no effect, or worsening of memory in response to phytoestrogens.

If one accepts the premise that an increase in BDNF would activate pro-survival signaling pathways and enhance memory, or at least prevent an age-associated decline in memory, as is supported by the literature (23-25), it would appear based on the results herein, that females would benefit from a diet rich in phytoestrogens if the diet or therapy regimen was initiated at a young or middle age. It is conceivable that an older female would still benefit in as much as the phytoestrogens increase hippocampal BDNF while having no
effect on cortical BDNF. Males, particularly older males, may have an increased risk of responding negatively to phytoestrogens given that our data showed that a diet rich in phytoestrogens caused a decrease in cortical BDNF in the older male mice and were at least unable to increase cortical BDNF in the young and middle-aged male mice. Males may still show improved hippocampal driven memory given that phytoestrogens increased BDNF in the hippocampus.

One caveat to making the assumption that an increase in BDNF would translate into improved cognitive function is the consideration that BDNF is first translated as a pro-peptide, and subsequently cleaved to the mature form. The pro-form of BDNF binds with higher affinity to the p75 neurotrophin receptor. This family of receptors contain a “death domain” within their cytosolic regions, and as such could lead to cell death upon activation by a pro-neurotrophin (29). Once pro-BDNF is cleaved into its mature form, it then has a higher affinity for the trkB receptor. Upon activation of this receptor, pro-survival signaling pathways such as the MAPK and PI3K pathways become activated and are associated with the promotion of cell survival (23). The BDNF quantification method used in our studies measures total BDNF. In this way we cannot distinguish between which form predominates, therefore must make an assumption that the mature form is the predominant one. We have made this assumption based on previous work in the lab which showed that when an increase in total BDNF occurred in the explants, they were protected from insult and that when the trkB receptor was blocked by the antagonist k252a, the explants were no longer protected by the increase in BDNF (25). Such results suggested to us that in fact, the mature form of BDNF increased, resulting in neuroprotection mediated by the trkB receptor.
In our initial data analysis, we were unable to discern a direct correlation between ER expression and the effects that phytoestrogens have on BDNF mRNA and protein expression. Out of a concern that the data from the middle-aged group might be skewing the other data, perhaps based in part, on a variation in hormone levels/cyclicity in females, we reanalyzed the data to compare the young and old groups only. Based on this analysis we were able to speculate which receptors and promoters may be driving the effects of phytoestrogens in the different brain regions. The ER profile for the female cortex is such that ER-beta mRNA expression decreases in the old relative to the young. Phytoestrogens did not affect BDNF mRNA levels in young or old females but did cause an increase in BDNF protein, presumably due to an increase in translation, in the young females but not in the old animals. Because the absence of protein increase in the old animals coincides with the age at which we observed a decrease in ER-beta, we speculate that ER-beta is the mediator of phytoestrogen induced translation of BDNF. In addition, we believe that the transcriptional effects of phytoestrogens in the cortex are mediated by ER-beta acting at an ERE. This is based on the fact that Paech et. al. reported that when a ligand, classified as an ER partial agonist acts through ER-beta at an ERE site, there is no induction of transcription (30). The female hippocampus differs from the cortex in that ER-alpha and not ER-beta decreases in the old relative to the young. The effects of phytoestrogens in the female hippocampus are such that BDNF mRNA is decreased in the young but increased in the old. Additionally, phytoestrogens increased BDNF protein in both young and old. This data is consistent with our theory that ER-beta is mediating translation, or the increase in BDNF protein, as ER-beta levels do not change with age and the protein increase observed in young remains in the old. We believe that the transcriptional effects of phytoestrogens in
the female hippocampus may be mediated by ER-beta acting at an AP1 site. We base this idea on the fact that ligands which are classified as ER partial agonists which act via ER-beta, acting through an AP1 site induce transcription, albeit with lower efficiency than estradiol itself (30). In young mice where estradiol levels are presumed higher than those in old mice, we presume that the phytoestrogens are acting as a partial agonist and competing with estradiol, thereby decreasing transcription. In the old mice in which estradiol levels presumed to be lower, and competition is less likely to occur, the phytoestrogens act as a partial agonist to increase transcription. We are therefore proposing that phytoestrogens, bound to ER-beta, act via different promoters in different brain regions. In support of this theory, differential activation of promoters has been documented in MCF-7 cells (31).

The data obtained for the males in this study does not fit into our proposed model. However, there is much unknown with respect to the aged male brain. For example, we are unable to predict what the relative amounts of estradiol are in the male brain as aromatization of testosterone with advanced age has not been studied in detail. Similarly, we do not know what the contributions of androgens and their receptors might have with respect to phytoestrogens in the brain. Additionally, dihydrotestosterone (DHT), which was previously believed to be a “pure” (i.e. non-aromatizable) androgen, can be metabolized to 5alpha-androstane-3beta,17-beta-diol (3beta-diol) which is a known ER-beta selective agonist (32-34). The presence of such an ER-beta selective ligand may alter the relative ratio of available ER-alpha and ER-beta. Indeed, a recent study has found that the ratio of ER-alpha and ER-beta is important in synaptic plasticity (35). The same study also showed that estradiol-induced induction of BDNF was mediated through ER-beta (35). Therefore,
there are considerable differences in the male and female brain that make it difficult to explain the mechanisms of phytoestrogens fully based solely on the data presented here. Nevertheless, we have established the overall effect that phytoestrogens have on BDNF in the cortex and hippocampus of male mice. That information in itself is valuable in predicting a functional outcome of dietary phytoestrogens with respect to cognition.

Worth consideration, too is the possibility that there are other players involved in phytoestrogenic effects in the brain. One potential candidate for influencing the outcome is the insulin like growth factor receptor (IGFR). Studies have shown that blocking the IGF-1 receptor completely abolishes genistein’s neuroprotective effects in SK-N-SH cells (36) suggesting an essential role for IGFR signaling in genistein’s neuroprotective effects. It has been shown that in male mice, despite an increase in brain levels of IGF-1 receptors with age there is a significant decrease in receptor signaling in the older mice which was not a function of decreasing IGF levels (37). An age-associated decrease in functionality in this system could add value in explaining how the effects of phytoestrogens are differentially affecting mice of different ages.

The research presented herein has served to further our understanding of the potential mechanisms and possible limitations of the effects that phytoestrogens have on the brain. From the data obtained from our studies we have proposed a possible mechanism by which phytoestrogens regulate BDNF in females. Overall, we have found that phytoestrogens do regulate BDNF in the cortex and hippocampus. Because these are the two regions most involved in learning and memory, changes in BDNF in these brain regions could reflect changes in these critical brain functions. Going forward, such information should be considered when designing clinical studies designed to assess the
outcomes that phytoestrogens have on cognitive function. Given the fact that soy supplements are one of the top herbal supplements consumed by Americans today (38) and that it is consumed as a natural dietary component by millions of people in the U.S. and worldwide, it is essential that we definitively identify how phytoestrogens affect cognitive function. It is our hope that this work contributes to that understanding and helps to move the field forward.


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