Published in final edited form as:

Brain Res. 2007 July 23; 1159: 54–66.

A High Soy Diet Enhances Neurotrophin Receptor and Bcl-X_L Gene Expression in the Brains of Ovariectomized Female Rats

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Abstract

Estrogen is a powerful neuroprotective agent with the ability to induce trophic and antiapoptotic genes. However, concerns about negative overall health consequences of estrogen replacement after menopause have led to the adoption of other strategies to obtain estrogen's benefits in the brain, including the use of selective estrogen receptor modulators, high soy diets, or isoflavone supplements. This study sought to determine the ability of a high soy diet to induce neuroprotective gene expression in the female rat brain and compare the actions of soy with estrogen. Adult ovariectomized female rats were treated with 3 days of high dose estrogen or two weeks of a soy-free diet, a high soy diet, or chronic low dose estrogen. Different brain regions were microdissected and subjected to real time RT-PCR for neuroprotective genes previously shown to be estrogen-regulated. The principle findings are that a high soy diet led to the widespread increase in the mRNA for neurotrophin receptors TrkA and p75-NTR, and the antiapoptotic Bcl-2 family member Bcl-X_L. Immunohistochemistry confirmed increases in both TrkA and Bcl-X_L. Chronic low dose estrogen mimicked some of these effects, but acute high dose estrogen did not. The effects of a high soy diet were particularly evident in the parietal cortex and hippocampus, two regions protected by estrogen in animal models of neurological disease and injury. These results suggest that a high soy diet may provide beneficial effects to the brain similar to low dose chronic estrogen treatment such as that used for postmenopausal hormone replacement.

Keywords

Soy; isoflavone; diet; Bcl; neurotrophin; mRNA

Introduction

In numerous experimental models of neurodegeneration, the steroid hormone estrogen is a powerful neuroprotective agent. These include models of focal and global cerebral ischemia (McCullough and Hurn, 2003, Merchantaler, et al., 2003), trauma (Bramlett and Dietrich, 2001, Roof and Hall, 2000), Alzheimer Disease (Pike, 1999), and Parkinson's Disease (Callier, et al., 2001). Estrogen can also improve cognitive performance in rodents (Markowska and Savonenko, 2002), non-human primates (Rapp, et al., 2003), and humans (Duffy, et al., 2003, Sherwin, 2003). These experimental results have led to the hypothesis that the increase in neurodegenerative diseases in women after menopause results from the decrease in circulating estrogen. Although the numerous beneficial effects of hormone replacement may

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depend upon formulation, route of administration, and individual patient differences (Kuhl, 2005), replacement of estrogen through traditional hormone replacement therapy (Hupponen, et al.) is now controversial and contraindicated in many women (2005). Many are turning to alternative therapies for relief of menopausal symptoms and physiological benefits attributed to endogenous estrogen. One popular alternative is the consumption of foods or supplements containing soy or soy isoflavones (Newton, et al., 2002). However, little is known about the effects of soy supplements or diets high in soy upon the central nervous system.

Recent studies suggest that diets high in soy (Bu and Lephart, 2005, Lund, et al., 2001, Pan, et al., 1999, Pan, et al., 2001) or the administration of individual isoflavone phytoestrogens contained in soy (Trieu and Uckun, 1999) may have beneficial effects in the brain similar to that provided by estrogen. We (Schreihöfer, et al., 2005) and others (Burguete, et al., 2006) have recently demonstrated a neuroprotective effect of high soy diet in experimental stroke in the rat. The phytoestrogen genistein has also been shown to protect against focal ischemic brain damage in mice when administered intraperitoneally (Trieu and Uckun, 1999). Furthermore, ip genistein delays mortality in a mouse model of amyotrophic lateral sclerosis (Trieu and Uckun, 1999). *In vitro* soy isoflavones also provide protection from beta amyloid (Wang, et al., 2001, Zhao, et al., 2002), glutamate (Zhao, et al., 2002), and thapsigargin-induced apoptosis in primary cortical neurons (Linford and Dorsa, 2002). There is also increasing evidence for improvement of several types of cognitive function with high soy diets in women (Casini, et al., 2006, File, et al., 2005, File, et al., 2001, Kritz-Silverstein, et al., 2003) and rodents (Lee, et al., 2004), however, these effects remain controversial (Kritz-Silverstein, et al., 2003, Lee, et al., 2005).

As with estrogen, there are several potential mechanisms for neuroprotection by soy isoflavones. Soy isoflavones can bind and activate estrogen receptor-dependent transcription, activate growth factor-related intracellular signaling, act as antioxidants, and reduce inflammation (Lephart, et al., 2004). However, the mechanisms of actions of dietary levels of soy isoflavones *in vivo* are not clear. In the present study, we hypothesized that a high soy diet would increase the expression of neuroprotective genes in the ovariectomized female rat brain. We selected genes that have previously been shown to be estrogen-regulated in the rat brain and examined transcriptional regulation in microdissected brain regions using real time RT-PCR and distribution of a subset of these genes using immunohistochemistry. Because the *in vivo* regulation of neurotrophic genes such as BDNF (Gibbs, 1998, Gibbs, 1999, Jezierski and Sohrabji, 2000, Solum and Handa, 2002), TrkA (Gibbs, 1998, McCarthy, et al., 2002), NT4 (Jezierski and Sohrabji, 2000), and IGF-1 (El-Bakri, et al., 2004) by estrogen appears to be both region and sex specific, soy actions were compared with estrogen in this study. The cerebral cortex and the hippocampus were examined because these regions are protected from injury by estrogen. Because soy isoflavones are reported to be selective for estrogen receptor β (Kuiper, et al., 1998), we also examined the dorsal hypothalamus, encompassing the medial preoptic area and paraventricular nucleus, which are regions with relatively high ER β expression. (Shughue, et al., 1996).

Results

Physiological parameters

Physiological parameters and serum hormone levels in ovariectomized (OVX) rats are summarized in Table 2. All rats were placed on a soy-free, isoflavone reduced (IF) diet. Two week low estradiol treatment, which was designed to mimic low physiological levels of about 20 pg/ml (IFE group), resulted in a significantly lower body weight compared to rats treated with placebo (IFP group). Low dose hormone treatment in the IFE group led to increased uterine weight and circulating estradiol (E2) compared to the IFP and rats fed a high soy diet for 2 weeks (SP group). There was no evidence of a uterotrophic effect of the high soy diet.

Short-term acute E2 injections designed to mimic proestrous levels reached 77.6 ± 9.7 pg/ml. Total serum isoflavone levels in the SP group were more than 50 times higher than in the IFP and IFE groups. Equol, a metabolite of daidzein, made up 85% of the circulating isoflavones. Daidzein and two additional metabolites, dihydrodaidzein and O-desmethyldangolensin made up the remaining 15%. In agreement with another study using female rats on high soy diets (Lund, et al., 2001), genistein was a minor component in plasma and was below detectable levels in all groups.

Soy and low chronic estrogen effects on gene expression

Estradiol has previously been shown to alter the expression of several neuroprotective growth factors and growth factor receptor genes (Amantea, et al., 2005). We examined the steady state mRNA levels of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), insulin-like growth factor 1 (IGF-1) and their receptors using real time RT-PCR. NGF expression was significantly reduced by both estrogen and soy in the ventral hypothalamus and BDNF expression was only altered by a soy-induced increase in the insular cortex (Figure 1). We saw no significant effects on NT3 (Figure 1) or IGF-1 (data not shown).

In contrast, growth factor receptors were significantly altered by estrogen and a high soy diet. In the parietal cortex, a high soy diet increased TrkA receptor mRNA 3-fold and p75-neurotropic receptor (p75-NTR) almost 2-fold (Figure 1). A high soy diet also increased TrkA and p75-NTR moderately in the hippocampus and p75-NTR in the hypothalamus (Figure 1). Low dose E2 did not mimic the effect of soy in the parietal cortex, increasing TrkA mRNA in the insular cortex and decreasing it in the hippocampus (Figure 1). E2 had a small, but significant, effect on hippocampal p75-NTR levels similar to soy (Figure 1). Interestingly, in the ventral hypothalamus both E2 and soy increased TrkA expression while reducing NGF (Figure 1). No significant changes in TrkB (Figure 1) or IGF1 receptor were observed (data not shown).

Changes in TrkA protein expression were examined by immunohistochemical staining and cell counting in the somatosensory, motor, and parietal cortex and hippocampus. Both a high soy diet and E2 led to significantly increased numbers of detectable TrkA-immunoreactive (TrkA-IR) cells in the cortex and hippocampus compared to the IFP group (Figure 2). Histological examination revealed most TrkA-IR cells to have a neuronal morphology with staining throughout the cell body in addition to punctate staining consistent with contacts from cholinergic projection neurons in the basal forebrain (Figures 2 and 4). In the septum itself, a similar pattern was found, with punctate staining present in addition to a more uniform staining of the cell bodies (Figure 2). TrkA-IR cells were present in the somatosensory and motor cortex and extended back into the parietal cortex with the greatest expression in layer V (Figure 3). In addition, many cells examined at high magnification appeared to have only punctate staining, suggesting that they did not express TrkA, but still had TrkA inputs. These cells were not counted. Fewer cells were observed in the insular and piriform cortex (Figure 3). TrkA-IR cell bodies were also present in the septum, diagonal band, substantia innominata, basal nucleus, and ventral pallidum (Figure 3). A few scattered cells were also observed in the thalamus and caudate (Figure 3). In the hippocampus, TrkA-IR cells were expressed most highly in the dentate gyrus and CA3 (Figures 3 and 4), but scattered cells were also present in CA1 and CA2 (Figure 3). The distribution of TrkA-IR cells did not appear to be different among groups, rather the total number of detectable cells changed.

Because p75-NTR expression in the absence of TrkA is often considered a signal for cell death (Casaccia-Bonnel, et al., 1999, Kalb, 2005, Nykjaer, et al., 2005), the localization of both receptors was examined in the cortex and hippocampus using immunofluorescence to determine whether p75-NTR expression was increased in cells not expressing TrkA. Compared to TrkA, which was readily detected in layer V of the cerebral cortex in agreement with previous

results (Miller and Pitts, 2000), few p75-NTR expressing cells were detected over background staining. However, when p75-NTR-IR cells were detected, they were also TrkA immunoreactive (Figure 4). Staining for p75-NTR in the cortex and hippocampus was less intense and lower density than staining in the diagonal band of Broca and medial septum, areas more recognized for p75-NTR expression in the adult brain, and no significant differences among groups in p75-NTR immunostained cells were observed in the cortex or hippocampus (mean = 18.5 ± 7.4 cells/mm²; data not shown).

Bcl family gene expression

The antiapoptotic members of the Bcl2 family, Bcl2, and Bcl-X_L are regulated by E2 *in vivo* and *in vitro* (Amantea, et al., 2005). Low dose E2 and a high-soy diet both significantly increased Bcl2 mRNA levels in the hippocampus, and low dose E2 significantly increased levels in the parietal cortex (Figure 3), but these changes were less than 50%. A high soy diet significantly increased Bcl-X_L in all areas except the insular cortex (Figure 3). Similarly, chronic low dose E2 increased Bcl-X_L mRNA in all areas examined (Figure 3). Immunohistochemical staining in the parietal cortex revealed a significant increase in Bcl-X_L positive cells in SP rats, but not IFE rats compared to IFP rats. Although a trend for increased Bcl-X_L cell number in the hippocampus was observed, this effect was not significant (Figure 4).

Effect of proestrous E2 levels

Because of the relatively small changes in gene expression in response to a high soy diet and low dose chronic E2, we sought to examine whether an E2 dosing regimen similar to the rise observed during proestrous would have a greater stimulatory effect on the neurotrophin receptors and antiapoptotic genes. Three days of E2 injection lead to plasma levels similar to proestrous. However, this higher dose did not result in greater changes in gene expression. In the parietal cortex, high E2 levels did increase TrkA mRNA to a level similar to that observed with a high soy diet (Figure 5). However, the effect was minimal in other regions. In contrast to high soy and low estradiol, high E2 suppressed the expression of the p75 neurotrophin receptor (Figure 5). In addition, high E2 did not mimic the effects of low E2 or high soy on Bcl2 and Bcl-X_L (Figure 5).

Discussion

The use of dietary soy and soy isoflavones for general health and as alternatives to traditional hormone replacement therapy after menopause is increasing (Newton, et al., 2002). Several lines of evidence suggest that dietary soy can have beneficial effects in peripheral tissues (Adlercreutz, 2002), but much less is known about potential benefits for the brain. In this study, OVX rats given a high soy diet for two weeks showed potentially beneficial increases in both BDNF and growth factor receptor gene expression in several brain areas. In addition, a high soy diet increased the levels of Bcl-X_L mRNA in several regions. In addition, changes in TrkA and Bcl-X_L gene expression were confirmed by immunohistochemical staining. Because phytoestrogens from dietary soy are considered weak estrogens with a preference for ER β (Kuiper, et al., 1998), we anticipated that a chronic low dose E2 treatment, similar to what might be used in low dose hormone replacement, would be more likely to have similar effects to soy. However, the results for soy were not fully recapitulated by either low dose chronic E2, nor acute E2 treatment designed to mimic proestrous levels in the rat, suggesting that the effects of soy in the brain cannot be ascribed purely to the estrogenicity of isoflavones. Although soy phytoestrogens have a binding preference for ER β and have been used as selective ligands, the use of ER β knockout mice has revealed only scattered ER β selective actions in the brain (Gundlah, et al., 2005; Patisaul, et al., 2003). In addition, although the effects observed in the present study may be attributable to ER β selectivity, the circulating isoflavone doses we

observed can readily activate ER α and ER β in neuronal culture (Schreihöfer, 2005). Both ER isoforms are present in cortex, hippocampus, and hypothalamus, whereas the insular cortex has lower levels (Kritzer, 2002; Laflamme, et al., 1998). Message and protein for both receptors is found in cortex and hippocampus, but at comparatively much lower levels than in hypothalamus (Shughrue, et al., 1997). One possible interpretation of our results is that TrkA, p75-NTR, and Bcl-X_L are under the influence of ER β in the cortex, whereas activation of ER α or both receptors with E2 leads to opposing effects or a predominant action of ER α . Future experiments with selective agonists, antagonists, or knock out animals would help to elucidate these possibilities.

Neither E2 nor soy had large effects on growth factor mRNA levels. BDNF was only stimulated 50% in the insular cortex by soy. Gibbs (Gibbs, 1999) noted 20–40% increases in BDNF mRNA levels in hippocampus and pyriform cortex but not olfactory bulb or frontal cortex in response to single proestrous level E2 injections, although protein levels in the hippocampus decreased (Gibbs, 1999). Jezierski and Sohrabji (Jezierski and Sohrabji, 2000) also observed increased BDNF mRNA and protein in the olfactory bulb, but decreases in the cingulate cortex. A stimulatory effect of E2 on BDNF mRNA and protein in the hippocampus has also been reported by several investigators (Singh, et al., 1995; Sohrabji, et al., 1995; Solum and Handa, 2002; Zhou, et al., 2005). Using Northern analysis, Pan et al. (Pan, et al., 1999) showed that both E2 and a high soy diet increase BDNF mRNA levels in the frontal cortex of retired breeder female rats. In contrast, the addition of soya to the diet of male hooded Lister rats reduced BDNF mRNA in situ hybridization signal in the hippocampus and cortex (File, et al., 2003). However, all of these reported changes are regionally distinct and of small magnitude, similar to the results of the present study.

Estrogen, but not soy, has been shown to increase NGF mRNA in entorhinal cortex (Bimonte-Nelson, et al., 2004) and hippocampus (Pan, et al., 1999), and we only observed a significant effect of E2 and soy in the ventral hypothalamus, a region of high ER expression. A reduction in NGF expression was associated with increased TrkA in this region, perhaps reflecting a compensatory mechanism. A previous report showed an increase in NT3 mRNA by estrogen in the entorhinal cortex and frontal cortex of aged ovariectomized rats (Bimonte-Nelson, et al., 2004), but NT3 was not affected by E2 or soy in the regions we examined. Finally, IGF-1 and IGF-1 receptors have been shown to cross-talk with E2 signaling pathways and colocalize with estrogen receptors in the rat brain (Cardona-Gomez, et al., 2001). Although E2 can increase IGF-1 binding in the rat brain (El-Bakri, et al., 2004), and increase IGF-1 mRNA levels in a hippocampal cell line (Shingo and Kito, 2003) and the primate frontal cortex (Cheng, et al., 2001), we saw no significant effect of either E2 or soy in the present study. Overall, E2 and soy had minimal effects on the steady state mRNA levels of the growth factors we examined, although some regional changes were observed.

In contrast to the growth factors, both E2 treatment and a high soy diet had significant effects on growth factor receptors in several brain regions. The NGF receptor TrkA was increased dramatically by high dose E2 and a high soy diet in the somatosensory, motor, and parietal cortex, with smaller, regionally different effects in other areas. This result agrees with other studies showing increased TrkA mRNA or protein levels in several brain areas in response to E2 treatment, including the septum (Gibbs, 1998), hippocampus (McCarthy, et al., 2002), and olfactory bulbs (Jezierski and Sohrabji, 2001). To our knowledge, this is the first demonstration of TrkA modulation by E2 or a high soy diet in the cortex. Although only no changes were seen in TrkB expression, the low affinity neurotrophin receptor p75-NTR was differentially regulated by E2 and soy. Low dose chronic estrogen and soy caused a small, but significant, increase in p75-NTR mRNA, but not protein, in the hippocampus. In contrast, high dose acute E2 significantly reduced expression. Previous studies have shown that both high physiological (Jezierski and Sohrabji, 2001) and supra-physiological E2 concentrations (Ping, et al., 2002)

also reduce olfactory bulb and frontal cortex p75-NTR protein expression in ovariectomized rats. Similar to low dose chronic E2, a high soy diet increased p75-NTR in all regions, with the largest increase in the parietal cortex.

Although IHC confirmed the increases in TrkA expression at the protein level, the expression of TrkA is lower in the adult cortex and hippocampus than during development and when compared to regions such as the medial septum. Nevertheless, because this increase can be observed at the protein level, it is likely to have physiological consequences. The distribution of TrkA-IR in the present study is similar to previous reports with numerous perikarya stained in the septum, substantia innominata, basal nucleus, and diagonal band (Sobreviela, et al., 1994). Previous investigators have shown extensive TrkA-IR in the cortex, particularly in layer V (Bruns and Miller, 2007, Bruns and Miller, 2007, Miller and Pitts, 2000, Saragovi, 2005), a result similar to what we have observed. In addition to labeling in perikarya, we also observed high intensity punctate staining in most TrkA-IR cells. This staining likely represents terminals of cholinergic projection neurons (Campenot and MacInnis, 2004) or neurons involved in paracrine signaling (Bruns and Miller, 2007).

In this study, a major site of neurotrophin receptor regulation was the somatosensory, motor, and parietal cortex, and a difference was observed between E2 and soy. Low dose E2 had no effect, but high dose E2 increased TrkA while at the same time decreasing p75-NTR. In contrast, a high soy diet significantly increased the expression of both receptors. Although p75-NTR is often considered a “death receptor,” and TrkA is considered a pro-survival receptor, recent studies have suggested a far more complex scheme (Casaccia-Bonnet, et al., 1999, Kalb, 2005, Nykjaer, et al., 2005). In particular, it is the apparent balance between TrkA and p75-NTR expression that may ultimately decide the fate of cells. The apoptotic actions of p75-NTR are thus most apparent when p75-NTR levels are high in the absence of Trk receptors. In this respect, both high dose E2 and soy lead to potentially pro-survival states in the parietal cortex. E2 increases TrkA and decreases p75-NTR, a classic pro-survival change in neurotrophin receptor expression. However, in the presence of high TrkA, p75-NTR can enhance the pro-survival effect of TrkA ligands (Casaccia-Bonnet, et al., 1999, Kalb, 2005, Nykjaer, et al., 2005). Thus, the combined up-regulation of TrkA and p75-NTR in the high soy rats can thus also be interpreted as a pro-survival state.

The observation that nearly all p75-NTR-IR cells in the cortex also express TrkA further supports a neurotrophic role for soy. Furthermore, although changes in p75-NTR mRNA were observed, the number of p75-NTR immunoreactive neurons in the cortex was low and not changed with treatment, suggesting that these mRNA differences may not be reflected in functional p75-NTR changes. However, p75-NTR immunoreactive cells were readily observed in the medial septum, diagonal band, and substantia innominata. The observation that we saw near 100% of p75-NTR-IR cells colocalized with TrkA is likely due to the intensity of our staining and the threshold for detection. Indeed, signal intensity for p75 was lower than that for TrkA. Thus, some p75-NTR-IR neurons may not have been detected. Miller and Pitts previously reported that 94% of TrkA-IR cells in the cortex also expressed p75-NTR (Miller and Pitts, 2000) and Sobreviela et al. observed similar colocalization in several brain areas (Sobreviela, et al., 1994). However, not all p75-NTR-IR cells expressed TrkA (Miller and Pitts, 2000).

Strengthening a pro-survival role for a high soy diet is our observation that the antiapoptotic Bcl-2 family member Bcl-X_L mRNA was upregulated by both low dose E2 and a high soy diet. Bcl-X_L immunoreactive neurons in the cortex were similarly increased by the high soy diet. This action was not shared by high dose E2. E2 has previously been shown to increase Bcl-X_L protein levels in hippocampal cultures (Pike, 1999) and differentiated PC12 cells (Koski, et al., 2004), but, to our knowledge, this is the first demonstration of a stimulatory

effect of chronic low dose E2 or soy on brain Bcl-X_L *in vivo*. Both pro- and antiapoptotic Bcl-2 family members have been shown to be estrogen-regulated. In the arcuate nucleus, Bcl-2 immunoreactivity is increased by both endogenous and exogenous E2 (Garcia-Segura, et al., 1998). E2 also increases Bcl-2 in primary cortical (Honda, et al., 2001) and hippocampal cultures (Nilsen and Brinton, 2003). *In vivo*, few studies have been able to demonstrate basal effects of E2 on Bcl-2 expression (Garcia-Segura, et al., 1998), but E2 does maintain or increase levels in response to injury *in vivo* as well as *in vitro* (Amantea, et al., 2005, Dubal, et al., 1999). Previous results show that genistein enhances Bcl-2 expression in response to injury in human cortical neurons, but has no effects on basal expression (Sonee, et al., 2004). Estrogens and soy can also modulate brain levels of pro-apoptotic Bcl-2 family products such as Bad (Bu and Lephart, 2005) in a region specific manner, but most effects on pro-apoptotic factors are only apparent in injury paradigms (Amantea, et al., 2005). In the present study, low dose, chronic E2 increased Bcl-2 mRNA in the parietal cortex and hippocampus, and soy had a small effect in the same areas. Thus, under basal conditions, both E2 and soy increase the expression of intracellular antiapoptotic factors in the rat brain.

Although the dietary soy-induced changes in gene expression observed in the present study are consistent with neuroprotection, they were not completely consistent with an estrogenic effect of soy. Alternatively, these changes in gene expression might be interpreted as a response to an apoptotic challenge, as high dose genistein can induce neuronal apoptosis *in vitro* (Linford, et al., 2001) and *in vivo* (Choi and Lee, 2004). However, this is an unlikely scenario. First, genistein was not found in the circulation of rats in this study. Second, daidzein is not harmful to neurons at high concentration *in vitro* (Linford, et al., 2001). Furthermore, in contrast to high dose genistein, low dose genistein, daidzein, and equol all have neuroprotective actions *in vitro* (Linford and Dorsa, 2002, Zhao, et al., 2002). Most notably, examination of apoptosis in the brain by TUNEL staining revealed no evidence of increased apoptosis in soy fed rats in the present study (data not shown).

The concentration of soy isoflavones achieved through the use of a standard rodent chow in this and other studies (4–7 $\mu\text{mol/L}$) greatly exceeds those observed in Asian populations (1–2 $\mu\text{mol/L}$) (Nagata, et al., 2002, Pumford, et al., 2002), and may not reflect potential benefits that could be achieved by diet. However, individuals with >4 $\mu\text{mol/L}$ circulating isoflavones have been observed (Nagata, et al., 2002), and standard supplement intake can also lead to such high circulating isoflavones even hours after ingestion (Maubach, et al., 2004, Setchell, et al., 2003). Furthermore, although the major circulating isoflavone in this study was equol, equol levels in humans are quite variable and depend upon the ability to convert daidzein in the microflora of the gut (Atkinson, et al., 2005). Thus, it will be clinically important to know whether changes observed in the present study are dependent upon specific isoflavones.

The results of the present study suggest that a diet high in soy leads to significant increases in mRNA levels of neuroprotective genes in several brain areas including the parietal cortex and hippocampus. In support of this notion are recent reports of neuroprotective effects of genistein in an *in vivo* model of amyotrophic lateral sclerosis (Trieu and Uckun, 1999) and focal cerebral ischemia (Burguete, et al., 2006, Schreihöfer, et al., 2005). Whether soy isoflavones provide neuroprotection through estrogen receptor-dependent mechanisms is not clear; however, *in vitro* sub-micromolar concentrations of isoflavones can mimic some of estrogen's transcriptional effects in neurons (Schreihöfer, 2005). Alternatively, the vascular, antioxidant, or actions on other receptor types may underlie these dietary effects (Lephart, et al., 2004, Ricketts, et al., 2005). Although the potentially neuroprotective changes in gene expression induced by a high soy diet are not fully recapitulated by estrogen treatment, they do suggest that a high soy diet can have beneficial effects on the brain and may be able to provide postmenopausal women with a neuroprotective alternative to traditional hormone replacement therapy.

Experimental Procedures

Animals and treatments

All animal experimentation was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The Medical College of Georgia Institutional Animal Care and Use Committee approved all animal protocols. Seven to eight week old female Sprague Dawley rats (225–250 g; Harlan) were acclimated for one week in the animal facility on a 12:12 h light dark cycle. All rats were placed on a soy-free, casein-based custom isoflavone-free diet (Zeigler Brothers) that was macro- and micro-nutrient matched to the high soy diet used in this study (Lephart, et al., 2002). One week later, rats were bilaterally ovariectomized (OVX) under halothane anesthesia and randomly placed into one of three groups: isoflavone-free diet + placebo (IF-P), isoflavone-free diet + estradiol (IF-E), or high soy diet + placebo (SP). At the time of OVX, IF-E rats were implanted with subcutaneous 21-day time-release pellets containing 0.05 mg 17 β -estradiol (Innovative Research of America), and IF-P and SP rats received placebo pellets. After implantation of pellets, rats in the SP group were switched to a high protein soy-based rodent diet (Teklad 8604) while IF-P and IF-E rat remained on the soy-free diet. Other investigators have shown that this diet, with soy as the sole protein source, contains 400–600 μ g soy isoflavones/g (Lund, et al., 2001, Lund, et al., 2001, Setchell, 2001). In separate acute studies, rats were placed on an isoflavone-free diet for one week, ovariectomized, and randomized into an oil or estrogen group. Rats remained on the isoflavone-free diet for the remainder of the study. Eleven days after OVX, rats received daily subcutaneous injections of either 17 β -estradiol (200 μ g/kg) in sesame oil or oil alone for 3 days. The two-week soy regimen was chosen because we have previously shown neuroprotection against stroke using this regimen (Schreihöfer, et al., 2005). Similarly, the timing of the estrogen treatment was based on previous stroke studies where lower doses (s.c.) are effective over treatment periods of 7–10 days and higher doses are effective over shorter time intervals (Gibson, et al., 2006). Furthermore, differences in stroke size can be observed over the time course of a four-day estrous cycle in the rat and these differences correlate with estrogen levels (Carswell, et al., 2000). Six hours after the final injection, rats were sacrificed as explained below.

Two weeks after OVX, all rats were deeply anesthetized with urethane (1.7 g/kg ip), transcardially perfused with cold sterile saline, and decapitated with a guillotine. The brain was rapidly removed, placed in ice-cold sterile saline for 2 minutes and cut into seven 2 mm coronal sections in a brain matrix (Braintree Scientific) starting at the frontal pole. Slices were placed in RNALater (Ambion) at 4° C. At the time of sacrifice, the uterus of each rat was removed and weighed wet as an assessment of hormone treatment. Trunk blood was collected, and serum was separated by centrifugation and stored at –80° C for later assessment of serum estradiol and isoflavone levels. Serum estradiol levels were determined in duplicate by ELISA using a commercial kit (Bio-Quant). All samples were run on one plate with an intra-assay CV of 2.4%. Serum isoflavone levels were determined at the University of Alabama, Birmingham core laboratory under the direction of Dr. Stephen Barnes using reversed-phase HPLC with an electrospray ionization interface and mass spectrometry (Barnes, et al., 1998). Serum from four animals per group was randomly selected for analysis prior to analysis of PCR experiments.

RNA collection and real-time RT-PCR

Total RNA was isolated from dissected brain slices using a commercial kit with a DNase treatment step to remove any DNA contamination (RNEasy, Qiagen). Slices used correspond to approximately –1.0 and –3.0 mm dorsal of Bregma (Paxinos and Watson, 1997). Areas dissected included dorsal parietal cortex at the intersection of the motor and sensory cortices, the insular cortex, the dorsal hippocampus including CA1-CA4 and the dentate gyrus (from –3.0 section only), the dorsal hypothalamus encompassing the PVN, and the ventral

hypothalamus encompassing the arcuate nucleus. RNA concentration was determined in triplicate using RiboGreen RNA-binding dye (Molecular Probes) and RNA was stored at -80°C until used. Total RNA (500 ng) was reverse transcribed with oligo-dT using a commercial kit (OmniScript, Qiagen). Following reverse transcription, the sample was diluted, and aliquots were stored at -80°C . Real-time RT-PCR was performed on 25 ng equivalents in triplicate on an Applied Biosystems (AB) 7500 Sequence Detection System using AB TaqMan Gene Expression Assays as summarized in Table 1. These commercially available assays use 5' Fam labeled probes and a non-fluorescent Minor Groove Binder. All assays, except Bcl-2, were specific to rat and crossed exon boundaries to ensure amplification of mRNA only. The assay for Bcl-2 was designed for mouse, but covers a region with 100% homology to rat Bcl-2. Serial dilutions of non-specific rat brain RNA were used to generate standard curves and ensure that each gene product was amplified with similar efficiency. Each gene was amplified separately using AB Universal Taq Master Mix with Amp UNG and Rox dye in 25 μL . Cycling conditions were the same for all gene products: 2 min 50°C , 10 min 95°C , and 45 cycles of two-step amplification with a 15 sec 95°C denaturing step and a combined annealing/amplification step at 60°C for 1 min. Each 96 well plate included amplification of RNA from 1 animal from each group in randomized fashion. Several gene products were amplified on each plate, including a GAPDH endogenous control gene. No significant differences in GAPDH expression were detected between groups overall or in each brain area analyzed independently. Threshold amplification cycle number (CT) data from multiple plates was combined using Applied Biosystems Relative Quantification (RQ) software (SDS1.2) and the $\Delta\Delta\text{Ct}$ method with GAPDH as the endogenous control. All data are expressed as mean fold change $\pm$ SEM.

Immunohistochemistry

Twelve additional rats (IFP, SP, IFE, $n = 4/\text{group}$) were treated as described above prepared for immunohistochemical detection of TrkA, p75-NTR, or Bcl-X_L. Two weeks after OVX, rats were deeply anesthetized with urethane and transcardially perfused with PBS (pH 7.4) followed by formaldehyde (4% in 0.1 mM phosphate buffer, pH 7.4). Brains were removed and stored in fixative for 24 h at 4°C . Coronal sections (30 μm) were cut through the forebrain using a Vibratome (The Vibratome Company). Every twelfth section through the parietal cortex (corresponding to approximately 0 to -2 mm Bregma) was processed for immunofluorescent detection with antibodies directed against TrkA (Chemicon, AB1577, 1:2,000) and p75-NTR (Chemicon MAB365, clone 192-IgG, 1:2,500) or Bcl-X_L (Santa Cruz, sc-7122, 1:200). Before use, free floating sections were washed with 50 mmol/L Tris-buffered saline, pH 7.4 (TBS) and blocked at room temperature for 1 h with 10% horse serum. The sections were then incubated for 24 hours at 4°C with the primary antibodies diluted in TBS with 10% horse serum and 0.1% Triton X-100. Sections were then rinsed with TBS/1% horse serum. For TrkA and p75-NTR, secondary antibodies were prepared in TBS/1% horse serum/0.1% Triton X-100. Sections were incubated for 1 h at room temperature in biotinylated donkey anti-rabbit IgG (1:400, Jackson Immuno) or donkey anti-mouse-IgG (1:400, Jackson Immuno) followed by 1 hour in streptavidin-conjugated Cy3 (1:1000, Jackson). Bcl-X_L immunostaining was revealed using donkey anti-goat IgG (1:400, Jackson Immuno) followed by 1 hour in streptavidin-conjugated Cy3 (1:1000, Jackson). For double labeling, after rinsing the TrkA sections, they were incubated for 1 hour with donkey anti-mouse-Alexa 488 (1:200, Molecular Probes) for detection of p75-NTR. Sections were rinsed with TBS, mounted on slides, and cover slipped with Krystalon (Electron Microscopy Sciences). Immunofluorescence was detected using an Olympus BX50 microscope with appropriate filters. Images were captured with an Optronics Magnifier SP digital camera. Cells were mapped using NeuroLucida software and quantified using Neuroexplorer (both Microbrightfield). For cortical cell counts, a 0.5 mm^2 box was drawn over one hemisection of parietal cortex starting 100 microns from the midline and adjacent to the ventral extent of the central sulcus. Two additional 0.5 mm^2 boxes were placed 100 microns dorsal and 100 microns lateral to the first forming a diagonal strip of

boxes from ventral-medial to dorsal-lateral through the cortex. An observer blind to treatments counted a total of 6 sections per rat. The total number of cells is expressed as cells/mm². For hippocampal counts, all cells within the hippocampal field on the same side were counted in CA1-CA4 and the dentate gyrus. The hippocampus was traced in each section and the total area was calculated by Neuroexplorer. Counts are expressed as cells/mm². TrkA distribution throughout the hemisphere was assessed by Neurolucida mapping. As controls, sections from one animal were also processed without the primary antibody, but with biotinylated secondary antibodies and streptavidin-Cy3. In addition, the double staining protocol was reversed such that TrkA was detected with a direct-tagged Alexa 488 secondary antibody, and p75-NTR was detected with a biotinylated secondary antibody and streptavidin-Cy3. Processing without primary antibodies resulted in no staining.

To determine the level of apoptosis in rat brains, 18 micron cryosections from separate groups of rats (IFP, IFE, SP) were subjected to Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling (TUNEL) using a commercial kit (Calbiochem). Sections were counterstained with methyl green and examined at three different rostro-caudal levels in each animal (n=3/group). Tissue from an animal that had undergone experimental stroke was used as a positive control for TUNEL staining.

Data analysis

Each gene and area was analyzed separately. In the diet experiment, differences among the three groups of rats were assessed by ANOVA with *post-hoc* comparisons between groups performed with Tukey-Kramer test using InStat (Graphpad). A difference of $P < 0.05$ was considered significant. In rats injected with estrogen or oil, unpaired t-tests were used to determine significant differences.

Acknowledgements

This work was supported by National Center for Complementary and Alternative Medicine grant 1R01AT001882 to D. A. Schreihöfer. Portions of this data have been presented previously at The Endocrine Society's 87th Annual Meeting, San Diego, 2005

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Figure 1. Soy and estrogen-induced changes in neurotrophic and neurotrophic receptor gene expression in the brain

Relative steady state mRNA levels determined by real time RT-PCR in the parietal cortex (Par Ctx), insular cortex (Ins Ctx), hippocampus (Hipp), ventral hypothalamus (Ven Hyp), and dorsal hypothalamus (Dor Hyp) in ovariectomized female rats on an isoflavone free diet (IFP, solid bars), an isoflavone free diet plus low dose estrogen (IFE, open bars), or a high soy diet (SP, hatched bars) for two weeks ($n=7$ /group). mRNA levels are normalized to the IFP group in each area. Bars represent mean $\pm$ SEM. * = significantly different ($p<0.05$) from IFP, and # = significantly different from IFE.

Figure 2. TrkA-IR in the rat brain

Graph: Number of immunoreactive TrkA cells in the dorsal cortex and hippocampus of IFP (solid bars), IFE (open bars), and SP rats (hatched bars, n=4/group). Bars represent mean $\pm$ SEM. * = significantly different (p<0.05) from IFP. Low power image shows TrkA immunoreactivity in the septum revealed with Cy3. High power image shows perikarya and punctate TrkA-IR in septal neurons.

Figure 3. TrkA immunoreactive cell distribution in the rat brain

Representative distribution of TrkA-IR cells at 4 coronal levels in an IFP rat. Coordinates are mm relative to bregma. The left side of each figure is an idealized adaptation from the Paxinos and Watson stereotaxic atlas (Paxinos and Watson, 1997). Abbreviations: Cg, cingulate cortex; M, motor cortex; S, somatosensory cortex; Ins, insular cortex; Pir, piriform cortex; CPu, caudate putamen; LV, lateral ventricle; cc, corpus callosum; LSI, lateral septal nucleus; MS, medial septal nucleus; VDB, ventral diagonal band; HVB, horizontal diagonal band; VP, ventral pallidum; AcbC, nucleus accumbens core; DEn, dorsal endopiriform nucleus; f, fornix; ac, anterior commissure; SI, substantia inomenata; MCPO, magnocellular preoptic nucleus; vhc, ventral hippocampa commissure; D3V, dorsal third ventricle; Thal, thalamus; ic, internal

capsule; B, basal nucleus; LGP, lateral globus pallidus; LH, lateral hypothalamus; RSA, retrosplenial agranular cortex; CA1, CA2, CA3 hippocampal fields; DG, dentate gyrus of the hippocampus; MDM, medial dorsal thalamic nucleus; sub, submedial thalamic nucleus; MGP, medial globus pallidus; Amyg, amygdala; Ect, entorhinal cortex.

Figure 4. Colocalization of TrkA and p75-NTR immunoreactive cells

Upper Figure: colocalization of TrkA and p75NTR in hippocampal CA3 and dentate gyrus and cortex. Staining appears as both diffuse cell body staining and punctate staining (arrowhead). Lower Figure: Low power images reveal colocalization of TrkA and p75NTR in the hilus of the hippocampus (arrowheads) of an IFP, IFE, and SP rat. Right column shows TrkA-IR in the CA3 region. TrkA was detected with Cy3, and p75NTR was revealed with Alexa 488.

Figure 5. Soy and estrogen-induced changes in Bcl2 and Bcl-X_L gene expression in the brain
Relative steady state mRNA levels determined by real time RT-PCR in the parietal cortex (Par Ctx), insular cortex (Ins Ctx), hippocampus (Hipp), ventral hypothalamus (Ven Hyp), and dorsal hypothalamus (Dor Hyp). Graphs represent ovariectomized female rats on an isoflavone free diet (IFP, solid bars), an isoflavone free diet plus low dose estrogen (IFE, open bars), or a high soy diet (SP, hatched bars) for two weeks (n=7/group). mRNA levels are normalized to the IFP group in each area, and bars represent mean $\pm$ SEM. * = significantly different (p<0.05) from IFP, and # = significantly different from IFE.

Figure 6. Soy and estrogen-induced changes in Bcl-X_L immunoreactivity in the brain
 Number of immunoreactive Bcl-X_L cells in the parietal cortex and hippocampus of IFP (open bars), IFE (solid bars), and SP rats (hatched bars, n=4/group). Bars represent mean $\pm$ SEM. * = significantly different (p<0.05) from IFP. Photomicrographs show representative staining for Bcl-X_L (arrows) in the cortex of an IFP, IFE, and SP rat.

Figure 7. Acute estrogen induced changes in neurotrophic receptor and Bcl gene expression in the brain

Relative steady state mRNA levels determined by real time RT-PCR in the parietal cortex (Par Ctx), insular cortex (Ins Ctx), hippocampus (Hipp) and dorsal hypothalamus (D. Hyp) in ovariectomized female rats on an isoflavone free diet injected with sesame oil (oil, solid bars) or 200 μ g/kg 17 β estradiol in oil (E2, open bars) for three days (n=6/group). mRNA levels are normalized to the oil group in each area. Bars represent mean $\pm$ SEM. * = significantly different ($p < 0.05$) from oil.

Table 1

Gene expression assays used

Gene Target	AB Assay number	Gene name
Bcl2	Mm00477631 m1	mouse Bcl2
BclXL	Rn00580568 g1	Bcl2-like1
BDNF	Rn00560868 m1	Brain derived neurotrophic factor
GAPDH		GAPD (GAPDH) Endogenous Control (VIC/MGB Probe, Primer Limited)
IGF1	Rn00570815 m1	Insulin-like growth factor 1
IGF1R	Rn00583837 m1	Insulin-like growth factor receptor 1
NGF	Rn00824646 m1	Nerve growth factor
p75-NTR	Rn00561634 m1	Neurotrophin receptor
NT-3	Rn00579280 m1	Neurotrophin 3
TrkA	Rn00582935 m1	Neurotrophic tyrosine kinase receptor, type 1
TrkB	Rn00820626 m1	Neurotrophic tyrosine kinase receptor, type 2

Table 2
Physiological parameters, serum estradiol, and serum isoflavone levels

	IFP	IFE	SP
Body Weight (g)	269±7	232±3 [*]	264±4
Uterine Weight (g)	0.18±0.03	0.59±0.18 [*]	0.19±0.04
Estradiol (pg/ml)	7.4±2.9	22.9±10.2 [*]	5.1±3.5
Total isoflavones (nmol/L)	114±121	71±35	6530±1982 [*]
Genistein (nmol/L)	0	0	0
Daidzein (nmol/L)	0	19±9	577±403 [*]
Dihydrodaidzein (nmol/L)	114±121	20±10	250±192
O-desmethylangolensin (nmol/L)	0	6±9	127±71 [*]
Equol (nmol/L)	0	23±9	5577±1626 [*]

IFP = isoflavone free diet + placebo (n=4); IFE = isoflavone free diet + estradiol (n=4); SP = high soy diet + placebo (n=4).

^{*} = Significantly different from other treatment groups ($P<0.05$).

[†] = significantly different from oil injected ($P<0.05$). ND = not determined.