Published in final edited form as:

Neurotoxicol Teratol. 2014 ; 41: 80–88. doi:10.1016/j.ntt.2013.12.004.

The effects of dietary treatment with S-equol on learning and memory processes in middle-aged ovariectomized rats

Steven L. Neese^{a,b,c}, Samantha L. Pisani^b, Daniel R. Doerge^d, William G. Helferich^d, Estatira Sepehr^d, Amar G. Chittiboyina^f, Sateesh Chandra Kumar Rotte^f, Troy J. Smillie^f, Ikhlas A. Khan^f, Donna L. Korol^{b,g}, and Susan L. Schantz^{a,b}

Steven L. Neese: stlneese@illinois.edu; Samantha L. Pisani: spisan2@illinois.edu; Daniel R. Doerge: daniel.doerge@fda.hhs.org; William G. Helferich: helferic@illinois.edu; Estatira Sepehr: estatira.sepehr@fda.hhs.gov; Amar G. Chittiboyina: amar@olemiss.edu; Sateesh Chandra Kumar Rotte: sckumarr@olemiss.edu; Troy J. Smillie: tsmillie@olemiss.edu; Ikhlas A. Khan: ikhan@olemiss.edu; Donna L. Korol: dlkorol@syrr.edu; Susan L. Schantz: schantz@illinois.edu

^aDepartment of Comparative Biosciences, University of Illinois at Urbana-Champaign. 2001 S. Lincoln Avenue, Urbana, IL 61802, USA

^bNeuroscience Program, University of Illinois at Urbana-Champaign. 505 South Goodwin Avenue, Urbana, IL 61801, USA

^cDepartment of Psychology and Neuroscience, Baldwin Wallace University. 275 Eastland Road, Berea, OH, 44017, USA

^dDivision of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration. 3900 NCTR Road, Jefferson, AR 72079-9502, USA

^eDepartment of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign. 905 S. Goodwin Avenue, Urbana, IL 61801, USA

^fNational Center for Natural Product Research, University of Mississippi, 3011 Thad Cochran Research Center, University, MS 38677, USA

^gDepartment of Biology, Syracuse University, 107 College Place, Syracuse, NY 13244, USA

Abstract

The use of over-the-counter botanical estrogens containing isolated soy isoflavones, including genistein and daidzein, has become a popular alternative to traditional hormone therapies. Menopausal women use these products as an aide in healthy aging, including for the maintenance of cognitive function. The safety and efficacy of many of these commercial preparations remains unknown. Previous research in our lab found that treatment of ovariectomized (OVX) female Long-Evans rats with genistein impaired working memory in an operant delayed spatial alternation (DSA) task and response learning in a plus-maze, but enhanced place learning assessed in the plus-maze. The present study further examined the effects of isolated isoflavones on working memory and place learning by treating middle-aged (12–13 month old) OVX female

© 2013 Elsevier Inc. All rights reserved.

Correspondence concerning this article should be addressed to Steven L. Neese, Department of Psychology and Neuroscience, Baldwin Wallace University. 275 Eastland Road, Berea, OH, 44017. sneese@bw.edu. Phone: 440-826-3712.

5. Conflict of Interest Statement

The authors have no potential conflicts of interest to report.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Long-Evans rats with *S*-equol, the exclusive enantiomer produced by metabolism of daidzein in the mammalian gut. *S*-equol binds selectively to ER β with an affinity similar to that of genistein but has low transcriptional potency. For DSA testing, *S*-equol at 1.94, 0.97 mg, or 0 mg (sucrose control) was orally administered to animals daily, 30 minutes before behavioral testing, and again both 4 and 8 hours after the first treatment. Rats were tested on the DSA task following the first, morning dose. For place learning, rats received 0.97 mg *S*-equol every 4 hours during the light portion of the cycle beginning 48 hours prior to behavioral testing (total exposure 8.7 mg *S*-equol). *S*-equol treatment was largely without effect on the DSA and place learning tasks. This is the first study to test the behavioral effects of isolated *S*-equol in OVX rodents, and shows that, unlike genistein or estradiol, repeated daily treatment with this isoflavone metabolite does not alter learning and memory processes in middle-aged OVX rats.

Keywords

equol; phytoestrogens; aging; executive function; place learning

1. Introduction

Traditional estrogen-based hormone therapies (HRT) continue to fall out of favor for use in peri- and postmenopausal women due to a variety of reported health consequences, including increased stroke and breast cancer risk and impairments in cognitive processes (see Craig et al., 2005; Narod, 2011; Renoux and Suissa, 2011 for recent reviews). In particular, findings from The Women's Health Initiative Memory Study (WHIMS) suggested that HRT has little effect on memory decline and may actually increase dementia risk in menopausal women (Craig et al., 2005; Zhao and Brinton, 2007). Menopausal women are now choosing to take alternative and complementary therapies marketed as "natural" treatments that offer the positive health effects of estrogens without the unwanted side effects. However, the claimed health benefits attributed to such botanical estrogens are largely unsubstantiated due to insufficient research in this important area. As a result, studies have been initiated in an effort to determine the efficacy of the isolated compounds in these over-the-counter supplements.

A variety of soy-based dietary supplements offer concentrated tablets containing isolated isoflavones, including genistein, daidzein, and/or glycitein, that possess estrogenic properties (Chua et al., 2004; Kostelac et al., 2003; Setchell et al., 2001). Importantly, the isoflavone content of these over-the-counter tablets can vary widely between manufacturers (Setchell et al., 2001); making it difficult to discern what, if any, active ingredients may aide in healthy cognitive aging. For instance, reports of the effects of several commercially available dietary soy-based supplements (with mixed isoflavone contents/concentrations) on learning and memory processes from randomized controlled studies in aging women vary, reporting no effect (Basaria et al., 2009; Fournier et al., 2007; Henderson et al., 2012; Ho et al., 2007; Kreijkamp-Kaspers et al., 2004), some improvement (Casini et al., 2006; Duffy et al., 2003; File et al., 2005; Gleason et al., 2009; Kritz-Silverstein et al., 2003; Santos-Galduroz et al., 2010), or even an impairment (Kritz-Silverstein et al., 2003). In addition to isoflavone composition, methodological differences in age at the time of treatment, previous HRT use, cognitive domain tested, and length of treatment likely contribute to the conflicting findings among published reports (see also Sumien et al., 2013). In contrast, treatments with mixed isoflavone content to young and aging ovariectomized (OVX) rats enhance performance on a variety of spatial learning and memory tasks (Lee et al., 2009; Monteiro et al., 2008; Pan et al., 2000, Pan et al., 2010; Sarkaki et al., 2008, 2009; Zhao et al., 2011). These limited results corroborate the oft-reported findings that estrogen-based treatments to OVX rats improve performance on tasks that tap the hippocampal memory

system in aging rodents (see Acosta et al., 2013; Frick, 2009; Hammond and Gibbs, 2011, but see also Chisholm and Juraska, 2013).

Equol is a metabolite of formononetin and daidzein first identified in equine urine, with later studies confirming its presence in rodent and human urine and plasma (Setchell and Clerici, 2010). Unlike the parent isoflavone, equol exists as both *R*- and *S*-enantiomers (Shor et al., 2012). In humans, *S*-equol is the exclusive enantiomer formed from daidzein by the gut microflora (Setchell and Clerici, 2010). Moreover, *S*-equol has a higher relative binding affinity for the classical nuclear estrogen receptors (ERs) than its parent molecule (Muthyala et al., 2004), with a ten-fold binding preference for ER β (ESR2) over ER α (ESR1) in vitro (Muthyala et al., 2004; Setchell et al., 2005). Interestingly, the fraction of Western adults able to convert daidzein to *S*-equol is low (20–35%) compared to Asian adults (50–60%: Arai et al., 2000; Atkinson et al., 2005; Rowland et al., 2000; Watanabe et al., 1998), while laboratory rodents tend to produce high levels of *S*-equol (Setchell et al., 2002, 2005; Setchell and Clerici, 2010). It has been suggested that the ability to produce *S*-equol is regulated to some extent by the diet as Asian populations tend to consume a larger amount of soy products than Westerners consume (Shor et al., 2012). Differences in *S*-equol production may contribute to the discrepancies reported in human memory studies making it difficult to determine what role, if any, *S*-equol may play in healthy cognitive aging.

Despite the availability and use of products with soy isoflavone mixtures, there is a lack of research assessing the effects of isolated *S*-equol treatment on memory processes in humans. Further, few studies have determined the *S*-equol production status when testing the efficacy of mixed isoflavone preparations to enhance learning and memory in human cohorts (Fournier et al., 2007; Gleason et al., 2009; Henderson et al., 2012; Kreijkamp-Kaspers et al., 2004), with only two studies examining if *S*-equol production contributed to cognitive change after treatment (Henderson et al., 2012; Kreijkamp-Kaspers et al., 2004). Cognitive outcome did not differ significantly between producers and nonproducers, but one study reported a nonsignificant trend ($p=0.08$), with *S*-equol producers having a better overall cognitive composite change score following 2.5 years of mixed isoflavone treatment (Henderson et al., 2012). Importantly, the authors from both studies stated that low statistical power (due to the small number of *S*-equol producers) could also have resulted in the lack of reported statistical difference (Henderson et al., 2012; Kreijkamp-Kaspers et al., 2004).

In rodents, one study found brain levels of equol to be elevated following dietary isoflavone exposure (Lund et al., 2001). In addition, studies employing systemic equol treatment have reported alterations in several CNS biomarkers and behaviors in rodents and non-human primates (Blake et al., 2011; Hamilton et al., 2011; Ma et al., 2010; Yoneda et al., 2011). Despite these findings, no studies to date have examined the cognitive effects of isolated *S*-equol in female rodents. Exposure to racemic equol as part of a mixed isoflavone treatment to young adult (6-month old) OVX mice restored spatial memory performance in a maze task to pre-OVX levels (Zhao et al., 2011), an effect similar to what is seen on this task following estradiol treatment to OVX rodents (see Frick, 2009). However, estrogen receptor sensitivities and distributions in brain can change across the lifespan, particularly in response to declining estradiol levels (Chakroborty and Gore, 2004; Han et al., 2013). Thus, to understand the health benefit or risk of isolated isoflavones, tests need to be conducted across later life epochs. To more accurately model the brain estrogen receptor milieu at menopause, the current subjects were middle-aged rats ovariectomized around the time of reproductive senescence.

This current study follows from our previous research that detailed differential effects of dietary treatment with the soy isoflavone genistein to OVX rats on two behavioral tasks, the prefrontally mediated operant delayed spatial alternation task (DSA), a test of working

memory (Neese et al., 2010a, 2012) and the hippocampally-mediated place learning task (Pisani et al., 2012a). In particular, we found that oral treatment with a 0.97 mg dose of genistein to middle-aged OVX rats 3 times daily impaired acquisition of the operant DSA task (Neese et al., 2012). We have reported similar deficits following chronic estradiol treatment to middle-aged OVX rats on this same task (Neese et al., 2010b, 2013; Wang et al., 2009). In addition, acute genistein treatment (0.485 mg dose every four hours for 48 hours) to young OVX rats impaired response learning performance, but enhanced place learning performance, bidirectional effects again consistent with those seen with estradiol treatment on these same tasks (Korol and Kolo, 2002; Pisani et al., 2012a). The purpose of this study was to determine if *S*-equol would produce effects similar to those seen with genistein and estradiol treatment on the operant DSA (exp. 1) and place learning (exp. 2) tasks in middle-aged OVX rats.

2. Methods

2.1. General Methods

2.1.1. Animals—Long-Evans retired breeders, 12–13 months of age, were obtained from Harlan (Indianapolis, IN) and maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All rats were OVX under isoflurane gas anesthesia. Prior to behavioral testing, rats were weighed daily and food was restricted to maintain body weight of 80–85% of their free-feeding body weights. Standard rodent diets contain soy phytoestrogens, including genistein and daidzein, which can influence performance on these memory tasks (see Neese et al., 2010b, 2012; Pisani et al., 2012a) so the rats in this study were maintained on a soy-free diet (AIN-93G modified to substitute corn oil for the soybean oil, TestDiet, Richmond, IN). This is a relatively low fat purified diet (7% fat and approximately 16% of calories from fat). Water was available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign and were in accordance with the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (National Institutes of Health, 2002), the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (National Research Council, 2003), and the *AVMA Guidelines for the Euthanasia of Animals* (Journal of the American Veterinary Medical Association, 2001).

2.1.2. S-Equol synthesis and verification—*S*-equol (Fig. 1) was synthesized at NCNPR (University of Mississippi) with a chemical and enantiomeric purity >98%. *S*-equol was then prepared in a pellet form, and delivered to the rats orally in 97 mg sucrose tablets containing either 0.5% *S*-equol (5AVF, TestDiet) or sucrose only (5TUT, TestDiet). The equol content of the tablets was verified using HPLC (see Doerge et al, 2000), and the tablets were found to contain at least 90% of the targeted dose (0.485 mg).

2.1.3. S-Equol blood level verification and dose—A pharmacokinetic study was performed prior to testing to establish blood levels resulting from oral treatment of *S*-equol to OVX rats. A single dose of 10 mg equol in a sucrose pellet was administered to each of 5 rats, corresponding to approximately 30 mg/kg body weight (bw). Serial blood samples were drawn prior to equol dosing and 0.25, 0.5, 1, 2, 4, 8, and 24 h after treatment. Serum levels of total (i.e., with treatment by a mixture of β -glucuronidase and sulfatase to hydrolyze conjugated forms) and aglycone (i.e., without enzyme treatment) equol were determined in 10 μ l (total) or 50 μ l (aglycone) aliquots by using LC/MS/MS, with a detection limit of 0.1 μ M for total and 0.008 μ M for aglycone (see Twaddle et al., 2002).

2.1.4. Uterine horn collection—Following the completion of behavioral testing, rats were overdosed with CO₂ (exp. 1) or sodium pentobarbital (75 mg/kg i.p., exp. 2) for collection of uterine horns, which were dissected from the peritoneal cavity and immediately measured and weighed.

2.2. Experiment 1

2.2.1. Animals—Forty-seven 13-month-old female Long-Evans retired breeder rats were housed in a temperature and humidity controlled room (22°C, 40–55% humidity) on a 12-hour reverse light-dark cycle (lights off at 8:30 am). Rats were pair-housed by treatment group in polysulfone cages (45 x 24 x 20 cm) with woodchip bedding. Beginning one week after OVX surgery, rats were weighed daily and food was restricted to maintain them at 85% of their free-feeding body weights. Operant training began three weeks following OVX and occurred once daily, six days/week during the dark phase of the light cycle. Rats were fed one hour after the daily test session was completed.

2.2.2. Exposure—Rats were divided into three treatment groups: sucrose control (4 sucrose tablets, n=15), low equol (2 *S*-equol tablets and 2 sucrose tablets, 0.97 mg, n=16), or high equol (4 *S*-equol tablets, 1.94 mg, n=16). Therefore, each rat received 4 tablets per treatment. Each rat was treated individually, three times daily on testing days (see also Klein et al., 2010), approximately 30 minutes prior to operant testing (see below), then again both 4 and 8 hours later. The low-dose group weighed (mean $\pm$ SEM in grams) 290.29 $\pm$ 6.41 and received 2.91 mg *S*-equol daily (approximately 10 mg/kg bw/d), while the high dose group weighed 305.67 $\pm$ 8.00 and received 5.82 mg *S*-equol daily (approximately 19 mg/kg bw/d). The sucrose-treated group weighed 302.86 $\pm$ 8.28. Each rat also received a single treatment on Sundays (non-testing days) to maintain circulating levels. Based upon the pharmacokinetic study (see above), oral treatment 30 minutes prior to testing resulted in initial peak serum levels during behavioral testing. Blood samples for the rats in the current study were collected after the end of operant testing, 30 minutes following the morning treatment dose, and analyzed using LC/MS/MS (see Twaddle et al., 2002). Group sizes were selected based on power analyses of similar studies previously conducted in this laboratory. A positive control group was not included in this study as we have replicated the impairing effect of 17 β -estradiol on this DSA task in OVX rats several times (see Neese et al., 2010b, 2013; Wang et al., 2008, 2009).

2.2.3. Operant Testing—Behavioral testing was conducted in standard automated operant chambers (Med Associates Inc., St. Albans, VT) housed in sound-attenuated wooden boxes. All of the test chambers had the same features and dimensions: 21.6 cm high with a 29.2 cm x 24.8 cm stainless-steel grid floor that rested just above a tray filled with woodchip bedding. Soy-free 45-mg food pellets (5TUL, TestDiet) were used as rewards and delivered via a pellet dispenser centered 2.5 cm above the floor on the operant panel. A pair of retractable response levers and a pair of stimulus cue lamps were positioned symmetrically on both sides of the pellet dispenser. The levers were positioned below the lights, 5.7 cm from the midline and 7.0 cm above the floor. The cue lights were located 5.7 cm above the levers. Each chamber also contained a Sonalert tone generator, a white noise generator, and a house light located on the back wall. Experimental contingencies were programmed using the Med-State behavioral programming language (Med-Associates, Vermont).

2.2.4. Response shaping and lever press training—Rats were trained to press the response levers by using an autoshaping program (Neese et al., 2010a, 2010b, 2013; Wang et al., 2008, 2009). Autoshaping test sessions were terminated after 100 food rewards were delivered or 60 min elapsed. Criterion for this condition was set at 100 lever presses in two

consecutive sessions. Following autoshaping, the rats were exposed to a continuous reinforcement (CRF) schedule in which the lever associated with reinforcement alternated following delivery of every 5th reinforcer to prevent the rats from developing a lever or side preference. This cycle of alternating levers terminated after 100 food rewards were received or 60 min had elapsed. A performance criterion of 100 reinforcers earned for three consecutive sessions was established for this condition.

2.2.5. Training Phases Prior to DSA—After lever press training, the rats were trained to alternate their responses prior to beginning testing on the DSA task (Neese et al., 2010a, 2010b, 2013; Wang et al., 2008, 2009). The initial training schedule began with cued alternation (CA) training in which a cue light was illuminated to indicate the correct lever on each trial. Each correct cued lever press was reinforced. The lever associated with reinforcement was not altered following an incorrect response, i.e. the “correct” lever continued to be the lever opposite of the lever associated with reinforcement in the most recent “correct” trial. A 0-sec delay was imposed between trials in this initial cued training phase as levers were retracted and extended (<0.15 sec) between trials. Rats were trained to a criterion of one session above chance defined as >60% correct presses. Next, a noncued alternation (NCA) task was presented where the cue light no longer indicated the correct lever, and both cue lights were illuminated when the levers were extended. Correct responses still consisted of alternating right and left lever presses. As for CA, the correct lever was again always the lever opposite of the one on the trial in which most recent “correct” lever press occurred. Again, a 0-sec delay was imposed between opportunities to press with the levers retracting and extending between presses. Each rat was trained for 10 consecutive sessions on the NCA phase.

2.2.6. Delayed spatial alternation (DSA) testing—The final alternation phase was the variable DSA task. Following each lever press, variable intertrial delays of 0, 3, 6, 9, or 18 sec were randomly imposed between opportunities to press (Neese et al., 2010a, 2010b, 2013; Wang et al., 2008, 2009). There were 40 trials at each delay and a total of 200 trials per session. Intertrial delays were randomly balanced within each session and any specific delay was not presented on more than three consecutive trials. Each animal was tested for 25 consecutive sessions. One day after completion of behavioral testing all rats were euthanized using CO₂.

2.2.7. Statistical Analyses—The behavioral data were analyzed using SPSS for Windows, Version 20.0. Treatment group was included in the analyses as a between subject factor and significance was set at $p < 0.05$. When appropriate, Tukey post hoc tests were run for pair-wise comparisons. Uterine horn weights and serum equol levels were analyzed with ANOVA, using treatment group as the between subjects factor.

For CA, cumulative errors across all sessions served as the main measure of learning, and was analyzed using ANOVA for treatment group and cohort. For NCA, the overall proportion correct across the ten sessions served as the primary measure of learning, and was analyzed using a 4 (treatment group) x 3 (cohort) x 10 (session) mixed ANOVA where session was a repeated measures factor. For DSA, the proportion correct across the 25 test sessions was first averaged across blocks of 5 test sessions to produce five 5-session test blocks. Proportion correct at each intertrial delay across the 25 test sessions was then analyzed using a mixed 3 (treatment group) x 5 (block) x 5 (delay) repeated measures ANOVA with block (1 - 5) and delay (0, 3, 6, 9, 18 sec) serving as repeated measures factors.

2.3. Experiment 2

2.3.1. Animals—Twenty-five 12-month-old female Long-Evans retired breeder rats were OVX three weeks prior to training. Rats were housed individually in clear polycarbonate cages with woodchip bedding on a 12-hour light-dark cycle. Beginning 10 days prior to behavioral testing rats were weighed daily and food restricted to 80–85% of their free-feeding body weights.

2.3.2 Exposure—Rats were assigned to one of three treatment groups: equol, estradiol, or sucrose control. To control for injections in the estradiol group and for exposure to the tablets, all rats received injections (s.c.) 24 and 48 h before testing and two 97 mg sucrose tablets orally every 4 h during lights on beginning 48 h prior to testing, corresponding to 48 h, 44 h, 40 h, 28 h, 24 h, 20 h, 16 h, 4 h and 30 min before the behavioral task (Fig. 2). The sucrose control group received 2 sucrose tablets (0% *S*-equol) and sesame oil injections. The equol treated group received 2 sucrose tablets containing 0.5% *S*-equol (0.97 mg) and sesame oil injections, for a total *S*-equol consumption of 8.7 mg over 2 days. The estradiol group received injections of 4.5 µg/kg 17β-estradiol benzoate (Sigma-Aldrich, St. Louis, MO) in sesame oil and 2 sucrose tablets. An estradiol group was included as a positive control in this study as we have previously only reported peripheral estradiol treatment to improve place learning in young adult (3 mo) or very old (24 mo) OVX rats (Korol and Kolo, 2002; Korol et al., 2007; Pisani et al., 2012a). Additionally, only a low equol exposure was used in this experiment as place learning was previously found to be modulated by low to moderate but not high levels of estrogens (Pisani et al., 2012a). Immediately after testing and approximately 2.5 h after the final sucrose or equol treatment, all rats were injected with an overdose of sodium pentobarbital (75 mg/kg, i.p.) and blood was collected for equol analysis using LC/MS/MS (see Twaddle et al., 2002).

2.3.3. Training apparatus and testing procedure—A plus-shaped black Plexiglas® maze with arms measuring 105 cm long, 13 cm wide, and 7 cm high was used for behavioral testing. Each arm contained a food boat with inaccessible sucrose pellets to minimize the use of odor cues during learning. The maze was placed in the center of the training room on a small table 70 cm above the floor. Two symmetrically placed floor lamps provided ambient lighting. The room contained many large (0.5–2 m) two- and three-dimensional extra-maze visual cues, such as high-contrast posters, hanging items, shelves with books, a door, a desk, etc. Cues were placed approximately 1–2 m outside the maze in equal densities on each wall of the training room.

Training took place in a single day during the light phase. Rats were placed in a clean holding cage and allowed to acclimate to the testing environment for 10 min. In this task, rats are required to learn the spatial location of the goal arm containing the sucrose reward using extra-maze cues. The start location was semi-randomly counterbalanced between east and west arms of the maze while the location of the goal arm remained constant. Each trial began by baiting the goal arm with sucrose pellets then placing the rat into the designated start arm. Rats had a maximum of 2 min to enter one arm to find the food reward. A rat was allowed to remain in the arm for 10 sec or until it turned to leave, and was then placed in the holding cage for a 30 sec intertrial interval. The maze was rotated between trials to reduce the use of intra-maze cues, and rats were trained to 100 trials. During the intertrial interval, any urine and fecal boli were removed and the maze thoroughly cleaned with dilute enzymatic solution (Alconox®, White Plains, NY) to remove olfactory cues.

2.3.4. Statistical Analyses—Trials to criterion and percent correct choices over 10 trial blocks were used to assess learning. Learning criterion was defined as 9 out of 10 correct choices, with at least six consecutively correct choices. Trials to criterion and uterine horn

data were analyzed via ANOVA, with treatment as the between subjects factor. Post-hoc comparisons between each treatment group were made using Fisher's PLSD tests. Percent accuracy over blocks of ten trials was plotted to generate learning curves, which were assessed using repeated-measures ANOVAs with percent correct choices as the dependent measure, trial block as the within subjects variable, and treatment as the between subjects variable. Unpaired t-tests were used to compare serum equol levels. Significance was set at $p<0.05$.

3. Results

3.1 Experiment 1

3.1.1. Animal attrition—Several rats were dropped from the study due to performance issues, treatment refusal, or sickness/death. For the sucrose control group, one rat was dropped prior to DSA testing as it refused to take all of its daily treatment doses. For the low equol group, one rat was dropped during the initial portion of DSA testing as it failed to reach 200 trials of testing for 5 consecutive testing sessions, and another was dropped during DSA testing as it refused to take all of its daily treatment doses. For the high equol group, two rats were dropped during NCA and DSA testing as they refused to take all of their daily treatment doses, while two additional rats were dropped because they became ill/died during DSA testing. Therefore, the final group sizes were as follows: sucrose control, $n=14$; low equol, $n=14$; high equol, $n=12$. Aside from refusal of treatment, rats that would not eat the sucrose or equol pellets did not exhibit other signs of morbidity or sickness as they ate, drank, and groomed normally and maintained their body weights.

3.1.2. Serum equol concentrations and uterine horn weights—A pharmacokinetic evaluation was conducted prior to behavioral testing to establish serum equol levels during the testing phase. A single oral dose of approximately 30 mg/kg bw produced average serum concentrations of total equol of 40–100 μM at approximately 30–60 min after dosing, of which approximately 0.2–0.6 μM was aglycone equol (i.e., the active form). Total and aglycone equol levels in serum followed a similar pattern with each reaching an initial peak by 1 h followed by a secondary peak in most rats by 8 h, and a return to near baseline at 24 hours. At peak concentrations, aglycone equol was less than 1% of the total serum equol. Previously we found that similar oral genistein treatment (approximately 10 mg/kg bw in a reward pellet) resulted in aglycone levels approximately 0.1 μM from 5 μM total genistein (i.e., ~2% aglycone, Neese et al., 2010a); therefore, to better parallel aglycone exposures from the genistein studies, the treatment doses of equol chosen here (i.e., 10–19 mg/kg bw) were similar or higher than those previously used for genistein to reflect the lower percentage of bioavailable aglycone equol in serum.

Treatment with *S*-equol produced elevated serum equol levels in both treatment groups at a single collection point near the maximum. This was revealed by a significant one-way ANOVA, $F(2,37)=29.85$, $p<0.001$. Tukey post hoc analyses found the high equol group to have significantly higher serum levels of total equol ($55.1 \pm 26.9 \mu\text{M}$) than did both the low equol and sucrose control groups 30 minutes after treatment, $p<0.05$ (Table 1). In addition, the low equol group ($28.4 \pm 4.8 \mu\text{M}$) also had higher serum levels of total equol than did the sucrose control group 30 minutes after treatment, $p<0.05$ (Table 1). Approximately 0.6% of the total serum equol was aglycone for the 0.97 mg-treated group, and 0.54% for the 1.94 mg-treated group. One rat from the sucrose control group had low but measurable total equol in its serum (all others were below the level of detection) but the aglycone level in this rat was below the detection limit. Uterine horn weights did not differ between treatment groups as revealed by a non-significant one-way ANOVA, $F(2,36)=0.166$, $p=0.848$.

3.1.3. CA and NCA Training—Treatment did not significantly alter errors committed during CA training. This was revealed by a non-significant ANOVA for treatment group, $F(2,37)=0.595$, $p=0.557$. In addition, performance accuracy during NCA training was not influenced by treatment group as indicated by a nonsignificant main effect of treatment group, $F(2,37)=0.180$, $p=0.836$ and nonsignificant treatment group x session interaction, $F(18,333)=1.082$, $p=0.377$. A main effect of session was uncovered, $F(9,333)=99.103$, $p<0.001$. The performance of all treatment groups improved across subsequent sessions of testing.

3.1.4. DSA Testing—Treatment did not alter performance during DSA training. This was revealed by a nonsignificant main effect of treatment group, $F(2,37)=2.167$, $p=0.129$ and a nonsignificant treatment group x block x delay interaction, $F(32,592)=1.293$, $p=0.229$ (Fig. 3). A significant block x delay effect was uncovered, $F(16,592)=23.654$, $p<0.001$. The performance of all treatment groups improved across subsequent sessions of testing, except following the longest intertrial delay.

3.2. Experiment 2

3.2.1. Serum equol and uterine horn weights—*S*-equol treatment significantly elevated serum levels of total equol over sucrose controls 2.4 hours after treatment, $F(1,14)=4.60$, $p<0.01$ (Table 2: equol group, $14.2 \pm 1.9 \mu\text{M}$; all rats in the sucrose control group had equol levels below the level of detection). Approximately 0.6% of the total serum equol was aglycone. Treatment had a main effect on uterine horn wet weight, $F(2,19)=78.54$, $p<0.001$, with estradiol treatment significantly increasing uterine horn size compared to control treatments ($p<0.001$), as expected. There was no uterotrophic effect of *S*-equol treatment ($p=0.589$).

3.2.2. Plus-maze testing—A significant main effect of treatment was found for trials to criterion, $F(2,19)=5.04$, $p=0.0176$. Estradiol administration enhanced learning, seen as a significant reduction in the number of trials to criterion as compared to sucrose control ($p=0.0056$, Fig. 4A). However, treatment with *S*-equol had no significant effect on place learning ($p=0.266$, Fig 4A).

A significant treatment x trial block, $F(18,171)=2.20$, $p=0.0049$, (Fig. 4B) and main effect of treatment, $F(2,171)=6.11$, $p=0.0089$, were uncovered for percent correct. Examination of learning curves shows that the estradiol group learned the task significantly more quickly than controls did, reaching high levels of accuracy significantly faster than the controls did halfway through training (Figure 4B). A main effect of trial block was also uncovered, $F(9,171)=46.62$. As Fig. 4B shows, all treatment groups made more correct choices as training progressed.

4. Discussion

The results of this study indicate there are no significant effects of either acute or chronic dietary treatment with the soy isoflavone metabolite *S*-equol on learning and memory processes in middle-aged OVX rats. The modulatory actions of estradiol on the tasks used here are well characterized, with elevated hormone levels impairing DSA performance but enhancing place learning. *S*-equol, which has been marketed as a safer botanical-based alternative to HRT, failed to mimic these cognitive effects of estradiol in a rodent model of the peri-menopausal woman.

4.1. Comparison to our previous rodent research

We have previously reported a working memory deficit following chronic treatment with the soy isoflavone genistein in aging OVX rats (Neese et al., 2010a, 2012). Specifically, treatment with a 0.97 mg dose of genistein 3 times daily impaired acquisition of this DSA task in middle-aged OVX rats (Neese et al., 2012), an effect similar to that seen following estradiol treatment (Neese et al., 2010b, 2013; Wang et al., 2009). Interestingly, the low equol treatment group in experiment 1 received an *S*-equol dose (0.97 mg, 3 times daily) equivalent to that of genistein in our previous study (see Neese et al., 2012), yet no change in performance was detected during DSA testing. These results were initially surprising given the fact that serum aglycone equol levels in both the low and high equol groups were similar to those seen following genistein treatment (Neese et al., 2012).

Estradiol is known to have opposing effects on cognition according to task requirements and the neural systems engaged, impairing performance on tests of executive function and egocentric learning and memory while enhancing spatial learning and memory. Results from experiment 2 demonstrate that in OVX middle-aged rats, estradiol improves place learning known to involve the hippocampus and related structures, similar to effects previously observed in young adult OVX rats (Korol and Kolo, 2002; Pisani et al., 2012a; Zurkovsky et al., 2006). This finding is consistent with those of others using different tasks showing that estradiol treatment enhances hippocampus-sensitive memory in middle-aged OVX rats (Bimonte-Nelson et al., 2006; Daniel et al., 2006; Rodgers et al., 2010; Talboom et al., 2008). As in the DSA task, genistein treatment also parallels the mnemonic effects of estradiol on the place task: acute treatment with 4.4 mg genistein over 48 hours enhanced place learning in young OVX rats (Pisani et al., 2012a). Other studies have reported that treatment with mixed isoflavones (which leads to exposure to daidzein, equol and genistein) tends to improve the performance of OVX rats on tasks that engage the hippocampus (Lee et al., 2009; Monteiro et al., 2008; Pan et al., 2000, 2010; Sarkaki et al., 2008, 2009; Zhao et al., 2011), yet treatment with *S*-equol failed to alter place-learning performance in this study. As expected, *S*-equol treatment also failed to stimulate ER α -dependent uterine horn growth, consistent with the results of others using similar and higher treatment doses administered by the oral route (Schwen et al., 2012; Selvaraj et al., 2004; Yoneda et al., 2011).

S-equol has a binding affinity for ER β comparable to genistein but has very weak transcriptional potency at ER β , with only 10% the activity of genistein in transcriptional assays (Muthyala et al., 2004). Additionally, *S*-equol loses its ER specificity at the level of gene expression, having similarly low transcriptional potencies at both ER α and ER β (Morito et al., 2001; Muthyala et al., 2004). Thus, there is a dissociation in the overall pharmacological potency of equol: the compound binds to ER β effectively but has weak, non-selective genomic actions. Importantly, we have shown that selective agonism of ER β with chronic DPN treatment paralleled the estradiol-induced deficit on this DSA task, whereas ER α agonism was largely without effect (Neese et al., 2010b). Agonism of either ER α or ER β can both dose-dependently enhance place-learning (Pisani et al., 2012b), although the genomic actions of ER β are most frequently implicated as critical for cognitive enhancement of hippocampus-sensitive tasks (Frick et al., 2010; Liu et al., 2008). Therefore, while genistein and *S*-equol have similar binding affinity for ER β , the distinct transcriptional activity of these two isoflavones at the classical nuclear ERs may underlie the differing outcomes on our learning and memory tasks.

Differences in phase II metabolism between equol and genistein may also contribute to the disparate cognitive outcomes measured between these isoflavones. Research has indicated a significantly lower fraction of bioavailable (aglycone) equol relative to genistein in plasma following dietary isoflavone exposure to mice (Allred et al., 2005), an effect confirmed in our pilot blood level study (see methods). Blood levels achieved by our dosing regimen

parallel those occurring in humans after consuming a high dose of S-equol and are about 10-fold greater than serum concentrations in humans after taking a therapeutic dose of 10–20 mg (Jackson et al., 2011; Setchell et al., 2005). The proportion of aglycone equol, the biologically active form, in serum was similar to fractions observed in humans and other animals following equol supplementation, with less than 1% of total equol existing in circulation as the aglycone form (Jackson et al., 2011; Schwen et al., 2012). Given the different pharmacodynamic and metabolic properties of equol, it is likely that genistein is the primary active component of mixed isoflavone treatments *in vivo*.

4.2 Relevance to human memory research

As discussed above, results from studies in human cohorts have reported inconsistent cognitive effects of treatments with supplements containing mixed isoflavones, with few assessing the S-equol production status of those involved. A set of published studies did determine S-equol production, but did not report a significant difference in cognitive performance between producers and nonproducers (Henderson et al., 2012; Kreijkamp-Kaspers et al., 2004). Due to the paucity of research and correlational nature of available data, it is difficult to reconcile the role of S-equol production status or the therapeutic potential of direct S-equol treatment to enhance learning and memory processes in aging women.

4.3 Adverse Effects

In experiment 1, four of the high dose S-equol treated rats were excluded from the study due to eventual refusal of treatment or sickness/death. There are discordant reports of the potential for treatment with S-equol to produce adverse effects in the human population. Treatment with SE5-OH [a commercially available product which contains S-equol as the major constituent of a mixed isoflavone preparation (Yee et al., 2008)], containing about 10–30 mg S-equol per dose, reported no adverse side effects (Aso et al., 2012; Setchell et al., 2009). However, other studies using SE5-OH (with about 10–40 mg S-equol per treatment) or direct S-equol treatment (10–320 mg) recorded adverse effects, including abdominal distention and nausea (Jackson et al., 2011; Jenks et al., 2012). Given that some of the rats dropped from the study refused to eat their treatments, stomach discomfort following chronic S-equol treatment could be a factor in their treatment refusal. The treatment doses used in experiment 1 were well below those used in a toxicity study in which 91-day treatments with 1000–2000 mg/kg doses of SE5-OH failed to alter mortality rates in female and male rodents (Yee et al., 2008), making it difficult to determine if the deaths of 2 rats in the high S-equol group were treatment related. Adverse effects were not observed in experiment 2.

4.4. Strengths of Study

A major strength of this study was that we assessed both acute and chronic daily oral dosing paradigms with S-equol in rats maintained on a soy-free diet. This model allows determination of the effects of isolated soy-based treatment, or lack thereof, as exposure to exogenous estrogens via the diet was minimized. Another strength of this study was that we used age-appropriate rats to model the perimenopausal woman (Cory-Slechta et al., 2001; Crofton et al., 2004; Li et al., 2008). In contrast, many studies use OVX young rats as a rodent model of human menopause when assessing the effects of a variety of isoflavones. Finally, we used two behavioral paradigms that tap different memory systems in the brain shown to be sensitive to a variety of estrogens (Korol and Kolo, 2002; Neese et al., 2010a, 2010b, 2012; Pisani et al., 2012a; Wang et al., 2008, 2009).

4.5. Summary and Conclusions

Overall, the results of this study suggest that dietary treatment with *S*-equol does not influence learning and memory processes in middle-aged OVX rats. Previous research from our laboratories found that working memory processes measured by the operant DSA task (Neese et al., 2010a, 2010b, 2012; Wang et al., 2008, 2009) and place learning assessed in a plus-maze (Korol and Kolo, 2002; Pisani et al., 2012a; Zurkovsky et al., 2006, 2007) are sensitive to both estradiol and dietary treatment with the soy isoflavone genistein. Equol has been proposed to be a valuable therapeutic compound to combat the detrimental effects of hormone loss in menopause, but had no discernible mnemonic effects in the current study. Therefore, equol does not appear to be a candidate treatment for the attenuation of cognitive loss associated with loss of female sex hormones during aging.

Acknowledgments

The authors would like to thank Mindy Howe and Suren Bandara for their help with OVX surgeries and daily animal care and John Katzenellebogen for reviewing this manuscript. This research was supported by the P50 AT006268 from ODS, NCAAM, and NCI (DLK, SLS, DRD, WGH). Steven Neese also received support from the National Institute of Environmental Health Sciences Grant T32 ES007326.

References

- Acosta JI, Hiroi R, Camp BW, Talboom JS, Bimonte-Nelson HA. An update on the cognitive impact of clinically-used hormone therapies in the female rat: Models, mazes, and mechanisms. *Brain Res.* 2013; 514:18–39. [PubMed: 23333453]
- American Veterinary Medical Association. AVMA guidelines on euthanasia. Am. Vet. Med. Assoc; Schaumburg, IL: 2007.
- Allred CD, Twaddle NC, Allred KF, Goepfinger TS, Churchwell MI, et al. Soy processing affects metabolism and disposition of dietary isoflavones in ovariectomized BALB/c mice. *J Agric Food Chem.* 2005; 53:8542–50. [PubMed: 16248551]
- Arai Y, Uehara M, Sato Y, Kimira M, Eboshida A, et al. Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. *J Epidemiol.* 2000; 10:127–35. [PubMed: 10778038]
- Aso T, Uchiyama S, Matsumura Y, Taguchi M, Nozaki M, et al. A natural *S*-equol supplement alleviates hot flushes and other menopausal symptoms in equol nonproducing postmenopausal Japanese women. *J Womens Health.* 2012; 21:92–100.
- Atkinson C, Frankenfeld CL, Lampe JW. Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. *Exp Biol Med.* 2005; 230:155–70.
- Basaria S, Wisniewski A, Dupree K, Bruno T, Song MY, et al. Effect of high-dose isoflavones on cognition, quality of life, androgens, and lipoprotein in post-menopausal women. *J Endocrinol Invest.* 2009; 32:150–5. [PubMed: 19411814]
- Blake C, Fabick KM, Setchell KD, Lund TD, Lephart ED. Neuromodulation by soy diets or equol: Anti-depressive & anti-obesity-like influences, age- & hormone-dependent effects. *BMC Neurosci.* 2011; 12:28. [PubMed: 21410981]
- Bimonte-Nelson HA, Francis KR, Umphlet CD, Granholm AC. Progesterone reverses the spatial memory enhancements initiated by tonic and cyclic oestrogen therapy in middle-aged ovariectomized female rats. *Eur J Neurosci.* 2006; 24:229–42. [PubMed: 16882019]
- Casini ML, Marelli G, Papaleo E, Ferrari A, D'Ambrosio F, et al. Psychological assessment of the effects of treatment with phytoestrogens on postmenopausal women: a randomized, double-blind, crossover, placebo-controlled study. *Fertil Steril.* 2006; 85:972–8. [PubMed: 16580383]
- Chakraborty TR, Gore AC. Aging-related changes in ovarian hormones, their receptors, and neuroendocrine function. *Exp Biol Med.* 2004; 229:977–87.
- Chisholm NC, Juraska JM. Factors influencing the cognitive and neural effects of hormone treatment during aging in a rodent model. *Brain Res.* 2013; 514:40–9. [PubMed: 23419893]

- Chua R, Anderson K, Chen J, Hu M. Quality, labeling accuracy, and cost comparison of purified soy isoflavonoid products. *J Altern Complement Med*. 2004; 10:1053–60. [PubMed: 15674001]
- Cory-Slechta DA, Crofton KM, Foran JA, Ross JF, Sheets LP, et al. Methods to identify and characterize developmental neurotoxicity for human health risk assessment. I: behavioral effects. *Environ Health Perspect*. 2001; 109 (Suppl 1):79–91. [PubMed: 11250808]
- Crofton KM, Makris SL, Sette WF, Medez E, Raffaele KC. A qualitative retrospective analysis of positive control data in developmental neurotoxicity studies. *Neurotoxicol Teratol*. 2004; 26:345–52. [PubMed: 15113596]
- Craig MC, Maki PM, Murphy DGM. The women's health initiative memory study: findings and implications for treatment. *Lancet Neurol*. 2005; 4:190–4. [PubMed: 15721829]
- Daniel JM, Hulst JL, Berbling JL. Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. *Endocrinology*. 2006; 147:607–14. [PubMed: 16239296]
- Doerge DR, Chang HC, Churchwell MI, Holder CL. Analysis of soy isoflavone conjugation in vitro and in human blood using liquid chromatography-mass spectrometry. *Drug Metab Dispos*. 2000; 28:298–307. [PubMed: 10681374]
- Duffy R, Wiseman H, File SE. Improved cognitive function in postmenopausal women after 12 weeks of consumption of a soya extract containing isoflavones. *Pharmacol Biochem Behav*. 2003; 75:721–9. [PubMed: 12895690]
- File SE, Hartley DE, Elsabagh S, Duffy R, Wiseman H. Cognitive improvement after 6 weeks of soy supplements in postmenopausal women is limited to frontal lobe function. *Menopause*. 2005; 12:193–201. [PubMed: 15772567]
- Fournier LR, Ryan-Borchers TA, Robison LM, Wiediger M, Park JS, et al. The effects of soy milk and isoflavone supplements on cognitive performance in healthy, postmenopausal women. *J Nutr Health Aging*. 2007; 11:155–64. [PubMed: 17435957]
- Frick KM. Estrogens and age-related memory decline in rodents: what have we learned and where do we go from here? *Horm Behav*. 2009; 55:2–23. [PubMed: 18835561]
- Frick KM, Fernandez SM, Harburger LL. A new approach to understanding the molecular mechanisms through which estrogens affect cognition. *Biochim Biophys Acta*. 2010; 1800:1045–55. [PubMed: 19913600]
- Gleason CE, Carlsson CM, Barnet JH, Meade SA, Setchell KDR, et al. A preliminary study of the safety, feasibility and cognitive efficacy of soy isoflavone supplements in older men and women. *Age Ageing*. 2009; 38:86–93. [PubMed: 19054783]
- Hamilton RT, Rettberg JR, Mao Z, To J, Zhao L, Appt SE, et al. Hippocampal responsiveness to 17beta-estradiol and equol after long-term ovariectomy: Implication for a therapeutic window of opportunity. *Brain Res*. 2011; 1379:11–22. [PubMed: 21241683]
- Hammond R, Gibbs RB. GPR30 is positioned to mediate estrogen effects on basal forebrain cholinergic neurons and cognitive performance. *Brain Res*. 2011; 1379:53–60. [PubMed: 21138734]
- Han X, Aenlle KK, Bean LA, Rani A, Semple-Rowland SL, et al. Role of Estrogen Receptor α and β in Preserving Hippocampal Function during Aging. *J Neurosci*. 2013; 33:2671–83. [PubMed: 23392694]
- Henderson VW, St John JA, Hodis HN, Kono N, McCleary CA, et al. Long-term isoflavone supplementation and cognition in women. *Neurology*. 2012; 78:1841–48. [PubMed: 22665144]
- Ho SC, Chan ASY, Ho YP, So EKF, Sham A, et al. Effects of soy isoflavone supplementation on cognitive function in Chinese postmenopausal women: a double-blind, randomized, controlled trial. *Menopause*. 2007; 14:489–99. [PubMed: 17308499]
- Jackson RL, Greiwe JS, Desai PB, Schwen RJ. Single-dose and steady-state pharmacokinetic studies of S-equol, a potent nonhormonal, estrogen receptor β -agonist being developed for the treatment of menopausal symptoms. *Menopause*. 2011; 18:185–93. [PubMed: 21341397]
- Jenks BH, Iwashita S, Nakagawa Y, Ragland K, Lee J, et al. A pilot study on the effects of S-equol compared to soy isoflavones on menopausal hot flash frequency and other menopausal symptoms. *J Womens Health*. 2012; 21:674–82.

- Klein MA, Nahin RL, Messina MJ, Rader JI, Thompson LU, et al. Guidance from an NIH workshop on designing, implementing, and reporting clinical studies of soy interventions. *J Nutr.* 2010; 140:1192S–1204S. [PubMed: 20392880]
- Korol DL, Kolo LL. Estrogen-induced changes in place and response learning in young adult female rats. *Behav Neurosci.* 2002; 116:411–20. [PubMed: 12049322]
- Korol, DL.; Zurkovsky, L.; Serio, SJ.; Decker, LA.; Gold, PE. Effects of age and task difficulty on estradiol enhancement of place learning. 37th Annual meeting for the Society for Neuroscience, Society for Neuroscience Abstracts; 2007. p. 95.20
- Kostelac D, Rechkemmer G, Briviba K. Phytoestrogens modulate binding response of estrogen receptors alpha and beta to the estrogen response element. *J Agric Food Chem.* 2003; 17:7632–5. [PubMed: 14664520]
- Kreijkamp-Kaspers S, Kok L, Grobbee DE, de Haan EHF, Aleman A, et al. Effect of soy protein containing isoflavones on cognitive function, bone mineral density, and plasma lipids in postmenopausal women. *JAMA.* 2004; 292:65–74. [PubMed: 15238592]
- Kritz-Silverstein D, Von Muhlen D, Barrett-Connor E, Bressel MAS. Isoflavones and cognitive function in older women: the Soy and postmenopausal health in aging (SOPHIA) study. *Menopause.* 2003; 10:196–202. [PubMed: 12792289]
- Lee YB, Lee KH, Sohn HS, Lee SJ, Kang IJ, et al. Effects of soy phytoestrogens on reference memory and neuronal cholinergic enzymes in ovariectomized rats. *J Med Food.* 2009; 12:64–70. [PubMed: 19298197]
- Li AA, Baum MJ, McIntosh LJ, Day M, Liu F, Gray LE Jr. Building a scientific framework for studying hormonal effects on behavior and on the development of the sexually dimorphic nervous system. *Neurotoxicology.* 2008; 29:504–19. [PubMed: 18502513]
- Liu F, Day M, Muniz LC, Bitran D, Arias R, et al. Activation of estrogen receptor-beta regulates hippocampal synaptic plasticity and improves memory. *Nat Neurosci.* 2008; 11:334–43. [PubMed: 18297067]
- Lund TD, West TW, Tian LY, Bu LH, Simmons DL, Setchell KD, et al. Visual spatial memory is enhanced in female rats (but inhibited in males) by dietary soy phytoestrogens. *BMC Neurosci.* 2001; 2:20. [PubMed: 11801187]
- Ma Y, Sullivan JC, Schreihof DA. Dietary genistein and equol (4', 7 isoflavandiol) reduce oxidative stress and protect rats against focal cerebral ischemia. *Am J Physiol Regul Integr Comp Physiol.* 2010; 299:R871–7. [PubMed: 20631292]
- Monteiro SC, Ben CBJ, Netto CA, Wyse AT. Ovariectomy impairs spatial memory: prevention and reversal by a soy isoflavone diet. *Metab Brain Dis.* 2008; 23:243–53. [PubMed: 18648916]
- Morito K, Hirose T, Kinjo J, Hirakawa T, Okawa M, et al. Interaction of phytoestrogens with estrogen receptors α and β . *Biol Pharm Bull.* 2001; 24:351–6. [PubMed: 11305594]
- Muthyala RS, Ju YH, Sheng S, Williams LD, Doerge DR, et al. Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of *R*- and *S*-equols and their differing binding and biological activity through estrogen receptors alpha and beta. *Bioorg Med Chem.* 2004; 12:1559–67. [PubMed: 15018930]
- Narod SA. Hormone replacement therapy and the risk of breast cancer. *Nat Rev Clin Oncol.* 2011; 8:669–76. [PubMed: 21808267]
- National Institutes of Health. Public Health Service Policy on Humane Care and Use of Laboratory Animals. NIH/Office of Laboratory Animal Welfare; Rockville, MD: 2002.
- National Research Council Institute for Laboratory Animals Research. Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. National Academy Press; Washington, DC: 2003.
- Neese SL, Wang VC, Doerge DR, Woodling KA, Andrade JE, Helferich WG, Korol DL, Schantz SL. Impact of dietary genistein on aging and executive function in rats. *Neurotoxicol Teratol.* 2010a; 32:200–11. [PubMed: 19945528]
- Neese SL, Korol DL, Katzenellenbogen JA, Schantz SL. Impact of estrogen receptor alpha and beta agonists on delayed alternation in middle-aged rats. *Horm Beh.* 2010b; 58:878–90.

- Neese SL, Bandara SB, Doerge DR, Helferich WG, Korol DL, Schantz SL. Effects of multiple daily genistein treatments on delayed alternation and a differential reinforcement of low rates of responding task in middle-aged rats. *Neurotoxicol Teratol.* 2012; 34:187–95. [PubMed: 21945133]
- Neese SL, Bandara SB, Schantz SL. Working Memory in Bisphenol-A Treated Middle-Aged Ovariectomized Rats. *Neurotoxicol Teratol.* 2013; 35:46–53. [PubMed: 23339879]
- Pan Y, Anthony M, Watson S, Clarkson TB. Soy phytoestrogens improve radial arm maze performance in ovariectomized retired breeder rats and do not attenuate benefits of 17beta-estradiol treatment. *Menopause.* 2000; 7:230–5. [PubMed: 10914615]
- Pan M, Li Z, Yeung V, Xu RJ. Dietary supplementation of soy germ phytoestrogens or estradiol improves spatial memory performance and increases gene expression of BDNF, TrkB receptor and synaptic factors in ovariectomized rats. *Nutr Metab.* 2010; 7:75.
- Pisani SL, Neese SL, Doerge DR, Helferich WG, Schantz SL, Korol DL. Acute genistein treatment mimics the effects of estradiol by enhancing place learning and impairing response learning in young adult female rats. *Horm Beh.* 2012a; 62:491–9.
- Pisani, SL.; Katzenellenbogen, JA.; Korol, DL. Effects of acute administration of ER-selective agonists on place and response learning in ovariectomized young adult female rats. 16th Annual meeting of the Society for Behavioral Neuroendocrinology; 2012b. p. 1.55
- Renoux C, Suissa S. Hormone therapy administration in postmenopausal women and risk of stroke. *Womens Health.* 2011; 7:355–61.
- Rodgers SP, Bohacek J, Daniel JM. Transient estradiol exposure during middle aged in ovariectomized rats exerts lasting effects on cognitive function and the hippocampus. *Endocrinology.* 2010; 151:1194–1203. [PubMed: 20068005]
- Rowland IR, Wiseman H, Sanders TAB, Adlercruetz, Bowey EA. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. *Nutr Cancer.* 2000; 36:27–32. [PubMed: 10798213]
- Santos-Galduroz RF, Galduroz JCF, Facco RL, Hachul H, Tufik S. Effects of isoflavone on the learning and memory of women in menopause: a double-blind placebo-controlled study. *Braz J Med Biol Res.* 2010; 43:1123–6. [PubMed: 20945036]
- Sarkaki A, Amani R, Badavi M, Moghaddam AZ, Aligholi H, et al. Pre-treatment effect of different doses of soy isoflavones on spatial learning and memory in an ovariectomized animal model of Alzheimer's disease. *Pak J Biol Sci.* 2008; 11:1114–9. [PubMed: 18819549]
- Sarkaki A, Badavi M, Aligholi H, Moghaddam AZ. Preventive effects of soy meal ($\pm$ isoflavone) on spatial cognitive deficiency and body weight in an ovariectomized animal model of Parkinson's disease. *Pak J Biol Sci.* 2009; 12:1338–45. [PubMed: 20128500]
- Schwen RJ, Hguyen L, Plomley JB, Jackson RL. Toxicokinetics and lack of uterotrophic effect of orally administered S-equol. *Food Chem Toxicol.* 2012; 50:1741–8. [PubMed: 22406328]
- Selvaraj V, Zakroczymski MA, Naaz A, Mukai M, Ju YH, et al. Estrogenicity of the isoflavone metabolite equol on reproductive and non-reproductive organs in mice. *Biol Reprod.* 2004; 71:966–72. [PubMed: 15151933]
- Setchell KD, Brown NM, Desai P, Zimmer-Nechemias L, Wolfe BE, et al. Bioavailability or pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J Nutr.* 2001; 131:1362S–75S. [PubMed: 11285356]
- Setchell KD, Brown NM, Lydeking-Olsen E. The clinical importance of the metabolite equol a clue to the effectiveness of soy and its isoflavones. *J Nutr.* 2002; 132:3577–84. [PubMed: 12468591]
- Setchell KD, Clerici C, Lephart ED, Cole SJ, Heenan C, et al. S-equol, a potent ligand for estrogen receptor β , is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. *Am J Clin Nutr.* 2005; 81:1072–9. [PubMed: 15883431]
- Setchell KD, Zhao X, Shoaf SE, Ragland K. The pharmacokinetics of S-(-)equol administered as SE5-OH tablets to healthy postmenopausal women. *J Nutr.* 2009; 139:2037–43. [PubMed: 19776178]
- Setchell KD, Clerici C. Equol: history, chemistry and formation. *J Nutr.* 2010; 140:1355S–62S. [PubMed: 20519412]
- Shor D, Sathyapalan T, Atkin SL, Thatcher NJ. Does equol production determine soy endocrine effects? *Eur J Nutr.* 2012; 51:389–98. [PubMed: 22366740]

- Sumien N, Chaudhari K, Sidhu A, Forster MJ. Does phytoestrogen supplementation affect cognition differentially in males and females? *Brain Res.* 2013; 514:123–7. [PubMed: 23415935]
- Talboom JS, Williams BJ, Baxley ER, West SG, Bimonte-Nelson HA. Higher levels of estradiol replacement correlate with better spatial memory in surgically menopausal young and middle-aged rats. *Neurobiol Learn Mem.* 2008; 90:155–63. [PubMed: 18485753]
- Twaddle NC, Churchwell MI, Doerge DR. High throughput quantification of soy isoflavones in human and mouse plasma using LC with electrospray-MS and MS/MS detection. *J Chrom B.* 2002; 777:137–43.
- Wang VC, Sable HJK, Ju YH, Allred CD, Helferich WG, Korol DL, Schantz SL. Effects of chronic estradiol treatment on delayed spatial alternation and differential reinforcement of low rates of responding. *Behav Neurosci.* 2008; 122:794–804. [PubMed: 18729633]
- Wang VC, Neese SL, Korol DL, Schantz SL. Chronic estradiol replacement impairs performance on an operant delayed spatial alternation task in young, middle-aged, and old rats. *Horm Beh.* 2009; 56:382–90.
- Watanabe S, Yamaguchi M, Sobue T, Takahashi T, Miura T, et al. Pharmacokinetics of soybean isoflavones in plasma, urine, and feces of men after ingestion of 60 g baked soybean powder (kinako). *J Nutr.* 1998; 128:1710–15. [PubMed: 9772140]
- Yee S, Burdock GA, Kurata Y, Enomoto Y, Narumi K, et al. Acute and subchronic toxicity and genotoxicity of SE-OH, an equol-rich product produced by *Lactococcus garvieae*. *Food Chem Toxicol.* 2008; 46:2713–20. [PubMed: 18554770]
- Yoneda T, Ueno T, Uchiyama S. S-equol and the fermented soy product SE5-OH containing S-equol similarly decrease ovariectomy-induced increase in rat tail skin temperature in an animal model of hot flushes. *Menopause.* 2011; 18:814–20. [PubMed: 21451423]
- Zhao L, Brinton RD. WHI and WHIMS follow-up and human studies of soy isoflavones on cognition. *Expert Rev Neurother.* 2007; 7:1549–64. [PubMed: 17997703]
- Zhao L, Mao Z, Schneider LS, Brinton RD. Estrogen receptor β -selective phytoestrogenic formulation prevents physical and neurological changes in a preclinical model of human menopause. *Menopause.* 2011; 18:1131–42. [PubMed: 21738079]
- Zurkovsky L, Brown SL, Korol DL. Estrogen modulates place learning through estrogen receptors in the hippocampus. *Neurobiol Learn Mem.* 2006; 86:336–43. [PubMed: 16979357]
- Zurkovsky L, Brown SL, Boyd SE, Fell JA, Korol DL. Estrogen modulates learning in female rats by acting directly at distinct memory systems. *Neurosci.* 2007; 144:26–37.

Highlights

Botanical estrogens are marketed to midlife women as an aide to healthy aging.

The efficacy of the botanical estrogen *S*-equol to alter cognition was assessed.

Middle-aged ovariectomized (OVX) rats were treated with *S*-equol.

Rats were tested on a prefrontal working memory or hippocampal place learning task.

S-equol treatment did not alter learning and memory on these tasks.

Figure 1.
Treatment schedule for subcutaneous estradiol injections and oral genistein dosing for experiment 2.

Figure 2.
(A–E) Proportion correct during DSA testing by intertrial delay. No treatment group differences were uncovered.

Figure 3.

(**A**) Trials to criterion for equol, estradiol, and sucrose control treatment groups (9 out of 10, at least 6 consecutive correct). Trials to criterion were lower following treatment with 4.5 μ g/kg estradiol ($*p < 0.05$). (**B**) Acquisition curves for equol, estradiol, and sucrose control treatment groups. Rats treated with estradiol acquired the task quicker than did the equol and sucrose treated groups.

Table 1

Serum equol levels collected 30 minutes after dosing for experiment 1.

Treatment Group	Total Serum Equol	Serum Aglycone Equol
Sucrose	0.01±0.01 *	< L.O.D.
Low Equol	28.4 ± 4.8 * (8.2–58.3)	0.1 ± 0.02 (0.06–0.28)
High Equol	55.1 ± 26.9 * (16.1–117.9)	0.3 ± 0.03 (0.14–0.58)

Total serum equol (in μM) 30 minutes after oral dosing was significantly different between all three treatment groups (Mean±SEM).

* Sucrose < low equol < high equol: $p < 0.05$.

Table 2

Serum equol levels collected 2.4 hours after dosing for experiment 2.

Treatment Group	Total Serum Equol	Serum Aglycone Equol
Sucrose	< L.O.D.	< L.O.D.
Low Equol	14.2 ± 1.9* (5.95–24.22)	0.1 ± 0.01(0.04–0.14)

Total serum equol (in μM) approximately 2.4 hours after oral dosing was significantly higher in the equol treated group (Mean $\pm$ SEM, * $p<0.05$). Serum equol in the sucrose treated group was below the level of detection (LOD).