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Impact of Dietary Genistein and Aging on Executive Function in Rats

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Abstract

Genistein is an estrogenic soy isoflavone widely promoted for healthy aging, but its effects on cognitive function are not well-understood. We examined the cognitive effects of once daily oral genistein treatment at two doses (approximately 162 µg/kg/day low dose and a 323 µg/kg/day high dose) in ovariectomized young (7 month), middle-aged (16 month), and old (22 month) Long-Evans rats. Operant tasks including delayed spatial alternation (DSA), differential reinforcement of low rates of responding (DRL), and reversal learning that tap prefrontal cortical function were used to assess working memory, inhibitory control/timing, and strategy shifting, respectively. At the conclusion of cognitive testing, brains were collected and relative densities of D1 and D2 dopamine receptor and dopamine transporter (DAT) were measured in the prefrontal cortex. On the DSA task, the high dose old group performed worse than both the high dose young and middle-aged groups. On the DRL task, the high dose of genistein resulted in a marginally significant impairment in the ratio of reinforced to non-reinforced lever presses. This effect was

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present across age groups. Age effects were also found as old rats performed more poorly than the young and middle aged rats on the DSA overall. In contrast, middle-aged and old rats made fewer lever presses on the DRL than did the young rats, a pattern of behavior associated with better performance on this task. Moreover, while DAT levels overall decreased with age, genistein treatment produced an increase in DAT expression in old rats relative to similarly aged control rats. D1 and D2 densities did not differ between genistein dose groups or by age. These results highlight the fact that aspects of executive function are differentially sensitive to both genistein exposure and aging and suggest that altered prefrontal dopamine function could potentially play a role in mediating these effects.

Keywords

genistein; aging; executive function; dopamine; prefrontal

1. Introduction¹

Currently, many products containing soy phytoestrogens are marketed to older women for the treatment of menopausal symptoms and/or to prevent age-related diseases such as osteoporosis, yet their effects on cognition remain unknown. The potential health benefits from soy consumption are presumed to be mediated by the soy isoflavones genistein, daidzein and the daidzein metabolite equol, that can each bind the estrogen receptor (ER) and initiate biological activity [32,56,83]. Because these products are plant-derived, they are advertised as safe and natural alternatives to hormone replacement therapy (HRT). However the potential for the active ingredients in these products to impact the aging process, and particularly cognitive decline during aging, is not clear.

Estrogens have long been associated with improved performance on cognitive tasks [reviewed in 15,20], and were widely used to curb age-associated cognitive decline in postmenopausal women until the Women's Health Initiative (WHI) brought to light potential risks associated with HRT. Studies assessing the effects of HRT on cognition during aging in women have yielded variable and often conflicting results, from improvements [8,16,30,49] to impairments [50,68]. As with HRT, soy consumption in women also has varied and inconsistent effects on cognitive function. Soy supplementation in postmenopausal women has been shown to improve performance on cognitive tasks that have been traditionally measured following HRT, including measures of mental flexibility, symbol recall, category fluency and nonverbal short-term memory [9,17,18,34]. In contrast, other studies found soy supplementation to impair performance on similar tasks of executive function [21], including verbal working memory [19]. Still other studies failed to find any soy induced changes in cognition on similar test batteries [26,33]. These results highlight the complex and often variable results reported in studies of the effects of estrogens on cognition. Several factors contribute to these differences in outcome including disparities in the length of time from the onset of menopause to initiation of treatment, type of HRT or form of soy supplement used, observational vs. randomized group assignment, and natural vs. surgical menopause. Additionally, the participants in the soy studies had varying backgrounds, with some coming from cultures that typically consume high levels of soy, which could influence the effects of exposure to additional soy.

In rodents, chronic oral exposures to dietary soy isoflavones have positive effects on performance in maze tasks that rely on intact hippocampus function. Ten-month oral dosing

¹Abbreviations: Delayed spatial alternation (DSA), Differential reinforcement of low rates of responding (DRL), 2, 3-bis(4-hydroxyphenyl) propionitrile (DPN), Prefrontal Cortex (PFC), Dopamine Transporter (DAT)

with soy phytoestrogens produced a dose-dependent improvement in performance of ovariectomized female retired breeders on a radial arm maze task [60]. Both 4-week and 10-week daily oral soy phytoestrogen supplementation (mixed isoflavones) also improved performance of ovariectomized female rats in a Morris water maze task [53,75]. These results mirror the cognitive enhancing properties of other estrogens on hippocampally mediated tasks [20].

The mnemonic enhancing effect of estrogens may be task- and brain region-specific. We have extensive findings suggesting behavioral impairments following estradiol exposure on tasks that tap brain regions outside of the hippocampus, including the striatum [31,96] and prefrontal cortex (PFC) [90,91]. One possible explanation for differential effects of estrogens across cognitive tasks is the heterogeneous distribution of ER across brain regions. The two known ER subtypes, ER α and ER β , have unique profiles of gene expression in estrogen responsive tissues [12,36], including brain regions such as the hippocampus and PFC [44,77,78]. Although both ER α and ER β are expressed in the PFC and the hippocampus, the ratio of ER α :ER β is more equal in the hippocampus, whereas very few ER α positive cells are found in the PFC [77]. Because soy phytoestrogens have higher potency and affinity for ER β [36], and because soy isoflavones have been found to accumulate in the frontal cortex [48], it is important to investigate the effects of dietary soy isoflavones on behavioral tasks that specifically engage the PFC.

Specific cognitive tasks have been identified that rely primarily on the PFC [e.g. 40,94] and thus can be used to assess the impact of soy phytoestrogens on prefrontal function. In particular, the medial PFC is critical for accurate performance on operant versions of the delayed match to sample and delayed alternation tasks that employ short delays (generally less than 15 seconds) [see 29,80], as well as tasks requiring timing and response inhibition [see 4,57]. We previously described cognitive deficits on two operant tasks mediated by the PFC following chronic estradiol treatment in young (3 month), middle-aged (12 month), and old (18 month) rats [90,91]. Chronic estradiol treatment impaired performance of female ovariectomized rats on an operant delayed spatial alternation (DSA) working memory task [90,91]. These deficits were seen at short delays (3–9 seconds), implicating modulation of PFC function in the behavioral effects. Estradiol-exposed rats were also less able to inhibit responding on a differential reinforcement of low rates of responding (DRL) operant task [90].

Importantly, the behavioral effects of estrogens can also be modulated by aging. Estradiol treatment prevented scopolamine induced deficits in the T-maze performance of middle-aged female rats (12–13 months), while no protective effect was seen in aged rats (20 months) [76]. A similar effect was seen in the Morris water maze, with estradiol treatment again enhancing performance in both young (5 months) and middle-aged female rats (16 months), again with no effect observed in aged rats (24 months) [84]. These studies suggest mnemonic enhancing effects of estradiol on hippocampally-mediated spatial tests in aging (16 months), but not aged (>20 months) rats. Aging also appears to be an important factor in ER expression and distribution, with more pronounced age-dependent decreases in ER β mRNA expression than ER α mRNA expression [93]. Therefore, brain areas and neural systems expressing primarily ER β , such as the PFC, as well as the cognitive functions regulated by these systems may be less responsive to estrogens and estrogenic substances, such as soy phytoestrogens, in old age.

There are many mechanisms through which estrogens can influence behavioral tasks that tap executive function. For example, performance on both the DSA and DRL tasks are sensitive to disruption of dopamine in the PFC [82,95], and estrogens are known to interact with the dopaminergic system [13,46]. Researchers have reported *in vivo* effects of dietary genistein

in the PFC, including an increase in apical dendritic spine density in the PFC of adult ovariectomized rats [47], and increased expression of choline acetyltransferase (ChAT) and several growth factor mRNAs in the frontal cortex of ovariectomized retired breeders [60,61]. However, we are not aware of any studies assessing *in vivo* effects of dietary soy on the PFC dopaminergic system.

The goal of this study was to determine if daily oral genistein treatment would produce behavioral impairments on several tasks of executive function similar to those we have previously described in ovariectomized female rats following chronic estradiol replacement [90,91]. Female rats continue to have significant circulating estradiol levels following ovariectomy [27,41], whereas women have very low circulating estradiol levels following menopause. Therefore, to better model the postmenopausal women and to allow comparisons with our previous estradiol replacement studies [90,91] ovariectomized rats were used in this study. The study was designed to model daily dietary genistein exposures humans might typically achieve taking commercially available soy supplements which are widely marketed to menopausal and post-menopausal women [1,14]. Working memory, inhibition and timing, and cognitive flexibility were assessed with an operant test battery we have used successfully to assess estradiol effects [90,91]. To determine if the effects of genistein varied across age, young, middle-age and old rats were tested on the operant battery. In addition, to determine the potential role of altered PFC dopamine in mediating genistein effects on these tasks, at the conclusion of behavioral testing brain tissue was collected and the expression of the dopamine receptors D1, D2 and dopamine transporter (DAT) in the PFC was measured.

2. Methods

2.1 Animals and Exposure

One hundred forty-four female Long-Evans rats were obtained from Harlan (Indianapolis, IN) in two cohorts spaced 6 months apart and were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). 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). 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 guidelines of the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* [58] and the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* [59].

Each cohort consisted of 24 young (7 months) virgin females, 24 middle-aged (16 months) and 24 old (22 months) retired breeders. Each age group was divided into three genistein treatment groups (sucrose control, low dose, high dose; see below) with 8 animals per age per dose in each of the two cohorts, or a total of 16 animals in each dose group at each age. Rats were pair-housed in standard plastic cages (45×24×20cm) with corn cob bedding. All rats were maintained on an AIN-93G soy-free diet (Harlan-Teklad, Madison, WI) to avoid additional exposure to dietary estrogens via the feed, with water available *ad libitum*. The diet was switched from standard rat chow to the AIN-93G on the day of ovariectomy surgery. No change in food intake or body weight was observed following the change in diet. Beginning one week after surgery, rats were food restricted to and maintained at 85% of their free fed body weights at the start of the restriction procedure. Rats were weighed daily and fed accordingly in order to maintain 85% free fed body weight. During behavioral testing, rats were fed one hour after the daily test session was completed. Testing began two weeks following ovariectomy and occurred once daily, six days/week during the dark phase of the light cycle.

2.2 Blood Isoflavone Analysis and Dosing

2.2.1 Genistein verification and blood levels—To achieve blood genistein levels comparable to those following soy supplementation in humans, rats were treated with 97 mg fruit flavored sugar pellets which contained 0.05% genistein (TestDiet, Richmond, IN, #1811494). The genistein content of the dosing pellets was tested using reverse phase HPLC with electrochemical detection [65], and they were found to contain ~94% of the targeted dose. Tail vein blood samples were collected in pilot rats prior to the beginning of the study to confirm blood isoflavone levels following dosing with genistein. Both total and aglycone genistein levels were determined in 10 μ l and 100 μ l aliquots of plasma, respectively, via LC/MS/MS [85]. The detection limit for a 10 μ l sample was 0.05 μ M.

2.2.2 Dosing—Control rats received two pellets containing sugar only. Two different genistein doses were used in this experiment: a 48.5 μ g genistein/day dose in which rats received one sugar and one 0.05% genistein pellet (approximately 162 μ g/kg/day, the “low dose” group), and a 97 μ g genistein/day dose in which rats received two 0.05% genistein pellets (approximately 323 μ g/kg/day, the “high dose” group). The range of doses per group was as follows (Mean $\pm$ SEM); Low dose: young (186.42 ± 2.88), middle-age (156.32 ± 4.93), old (155.82 ± 4.82); High Dose: young (382.70 ± 4.42), middle-age (329.74 ± 6.68), old (313.13 ± 10.42). Pilot research found serum genistein levels peaked at about one hour following dosing, and declined thereafter (data not shown). Therefore, in order to ensure that circulating genistein levels were elevated during behavioral testing, rats were treated one half hour before each daily testing session. Each rat was removed from its home cage, given its dose individually, and consumed its respective treatment dose within a few seconds. At the end of the study, blood was collected via tail vein one hour after treatment for analysis.

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 $\times$ 24.8 cm stainless-steel grid floor that rested just above a tray filled with corn cob bedding. Soy-free food pellets (45-mg Formula P, P.J. Noyes Inc., Lancaster, NH) were dispensed through a pellet dispenser centered 2.5 cm above the floor on the operant panel. Positioned symmetrically on both sides of the pellet dispenser were a pair of retractable response levers and a pair of stimulus cue lamps, one above each lever. The levers were 5.7 cm from midline and 7.0 cm above the floor and 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).

2.4 Response shaping and lever press training

Rats were trained to press the response levers by using an autoshaping program [90]. Autoshaping test sessions terminated after 60 minutes elapsed or 100 reinforcers were delivered, whichever occurred first. Criterion for this condition was set at 100 lever presses within a single session. Following autoshaping, the rats were exposed to a continuous reinforcement schedule in which the lever paired with reinforcement alternated following delivery of every fifth reinforcer. The purpose of this schedule was to strengthen the recently acquired lever press response and to prevent the rats from developing a lever or side preference. This cycle of alternating levers terminated after 100 reinforcers were received or 60 minutes had elapsed. A performance criterion of 100 reinforcers for two consecutive sessions was established for this condition.

2.5 Cued Alternation (CA), Non-cued Alternation (NCA), and Delayed Spatial Alternation (DSA)

After lever-press training, the rats were trained on a variable delay DSA task [90]. The sequence began with CA training in which a cue light indicated the correct lever on each trial. Each correct, cued lever press was reinforced. Levers were retracted and extended between trials. No delay was imposed between trials in the initial training phase (the time between retraction and extension of lever during the 0-second delay trials was <0.15 seconds). Rats were trained to a criterion of one session above chance defined as >60% correct presses. Next, a 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 with the levers retracting and extending between presses. Each rat was tested for 10 sessions on the NCA task. The final phase was the DSA task in which variable delays of 0, 3, 6, 9, or 18 seconds were imposed randomly between trials. There were 40 trials at each delay and a total of 200 trials per session. 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 sessions.

2.6 Differential Reinforcement of Low Rates of Responding (DRL)

On the day immediately following the completion of the DSA task, timing ability and inhibitory control were assessed using a DRL task. Only one lever, the right response lever, was used for the DRL condition. The lever remained extended throughout the session. The first two sessions of training consisted of a fixed-ratio 1 schedule in which every response was reinforced for 200 trials, or 90 minutes, whichever occurred first. Animals did not have to meet a performance criterion before moving on to the next stage of training. Sessions 3 and 4 of training consisted of a DRL-5 second schedule in which reinforcement was contingent on a minimum of at least 5 seconds separation between responses. If a response occurred within the 5-second window, the response timer was reset. Sessions 5 and 6 consisted of a DRL-10 second schedule. The rats were then tested for 30 sessions on a DRL-15 second schedule.

2.7 Spatial Reversal Learning

Immediately following completion of DRL extinction, cognitive flexibility and strategy shifting were tested using an operant spatial reversal learning task (spatial RL). The rat's consistent pressing of the lever associated with a particular spatial location (right or left lever) was reinforced. Equal numbers of rats from each treatment group and age were assigned to begin the spatial RL task with the left or the right lever paired with reinforcement. Each session began with the extension of both response levers into the testing chamber. Cue lights remained off throughout testing. A single press to the reinforcing lever resulted in the dispensing of a reward pellet, retraction of both levers, and the initiation of a 10 second inter-trial interval (ITI). A single press on the lever not associated with reward resulted in the retraction of the levers and the initiation of the ITI with no food reinforcement provided. Each session was terminated after 200 trials had been presented or 90 min had elapsed, whichever occurred first. A performance criterion of 85% correct for two consecutive sessions was established for this task. After the criterion was reached, the opposite lever became paired with reinforcement. Again, criterion for reversal back to the initial lever was set at 85% correct for two consecutive sessions. This cycle of reversing the lever associated with reinforcement continued until five spatial reversals were completed.

2.8 Western blotting

Following completion of the cognitive battery, rats were given an overdose of CO₂ and their brains were quickly removed. A subset of tissue samples (5–8 rats per treatment dose and

age group) were used for western blotting. The forebrain was marked with a cut at bregma and the brain was placed in liquid nitrogen and stored at -80°C . The medial PFC was isolated by cutting at A-P: 2.2–3.5 mm anterior to bregma; D-V: 3–5 mm from the cortical surface; M-L: 0–0.9 mm from the midline [66]) after thawing on ice.

Tissue was processed with a high-speed tissue homogenizer in T-PER Reagent (Bio-Rad, Hercules, CA) plus protease inhibitor (PI) (Roche, Nutley, NJ), and 400 μL of T-PER plus PI was added to each the tube. The protein lysate ($\sim 250\mu\text{L}$) was transferred to a clean tube via micropipette and centrifuged at $10,000 \times g$ for 5 minutes at 4°C and the supernatant transferred to a fresh tube.

Differences in D1 and D2 dopamine receptor and DAT densities were compared via Western blotting. A 20 μg aliquot of each protein sample was pipetted into chilled tubes with an equal volume of 2X Laemmli (Bio-Rad) sample buffer. Tubes were heated at $95-100^{\circ}\text{C}$ for 5 minutes and then centrifuged at $13,000 \times g$ for 5 minutes at 4°C . Tubes were then loaded onto a 10% polyacrylamide gel in the Mini PROTEAN 3 Cell (Bio-Rad) filled with cold 1X Tris/Glycine/SDS buffer. Electrophoresis was run at 200 volts for approximately 60 minutes or until proteins reached the bottom of the gel with a protein standard (Invitrogen Magic Mark XP, Carlsbad, CA). The proteins were transferred to a nitrocellulose membrane on a Criterion blotter (Bio-Rad) filled with cold 1X Tris/Glycine buffer. Transfer was set at 100 volts for 30 minutes. The blot was blocked in 5% milk 1X TBS for 1 hour at room temperature.

Membranes incubated in rabbit primary antibodies in 1X TBS/ 1% milk for 24 hours at 4°C . Primary antibodies included anti-D1 receptor antibody (1:1000, Calbiochem, San Diego, CA), anti-D2 receptor (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-DAT (1:1000, Chemicon, Temecula, CA). Mouse anti- β -actin antibody (1:100,000) was run in parallel to serve as an internal loading control (Abcam, Cambridge, MA). Secondary antibodies were added and membranes incubated one hour at room temperature (donkey anti-rabbit:HRP affinity purified antibody from Affinity BioReagents diluted 1:5000 and goat anti-mouse:HRP from Abcam diluted 1:100,000 in 1X TBS/ 1% milk). After incubation in secondary antibodies, membranes were rinsed in wash buffer and exposed with SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) for five minutes. The signal solution was prepared by mixing the two substrate components 1:1. The blot was then captured on autoradiography film (Kodak) and digitized using a scanner (HP). Optical densities were quantified using ImageJ software (NIH). Relative densities were normalized against β -actin protein.

2.9 Statistical Analyses

The behavioral data were analyzed via repeated measures ANOVA using SPSS for Windows, Version 15.0. Dose group, age, and cohort were included in the analyses as between subject factors and significance was set at $p < 0.05$. Blood genistein levels were analyzed via two-way ANOVA with dose and age as between subject factors. When appropriate, Tukey post hoc tests were run for pair-wise comparisons.

2.9.1 DSA Task—For CA, cumulative errors across all sessions served as the measure of learning, and were analyzed using a three-way, between-subjects ANOVA for dose, age and cohort. For NCA, the overall proportion correct across the ten sessions served as the primary measure of learning, and was analyzed using a 3 (dose) $\times$ 3 (age) $\times$ 2 (cohort) $\times$ 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 five test sessions to produce five 5-session test blocks. Proportion correct at each delay

across the 25 test sessions was then analyzed using a mixed 3 (dose) $\times$ 3 (age) $\times$ 2 (cohort) $\times$ 5 (block) $\times$ 5 (delay) repeated measures ANOVA with block (1 – 5) and delay (0, 3, 6, 9, 18 sec) serving as repeated measures factors. Response pattern analyses were used to assess the rats' tendency to repeat a correct or incorrect response. A win-stay error was defined as an incorrect response on the same lever that had been correct on the previous trial. A lose-stay error was defined as an incorrect lever press on the same lever that had been incorrect on the previous trial. Win-stay and lose-stay errors were analyzed separately using a mixed 3 (dose) $\times$ 3 (age) $\times$ 2 (cohort) $\times$ 5 (block) repeated measures ANOVA with block (1 – 5) serving as a repeated measures factor. .

2.9.2 DRL Task—For DRL, the total number of responses and total number of reinforcers delivered were the primary measures of learning. The data were averaged to yield six blocks of five sessions each. Performance was also assessed by examining the ratio of reinforced to non-reinforced lever presses. The total number of responses, reinforcers delivered, and ratio of reinforced to non-reinforced lever presses were analyzed using separate mixed 3 (dose) $\times$ 3 (age) $\times$ 2 (cohort) $\times$ 6 (block) ANOVAs where block (1–6) served as a repeated-measures factor.

Inter-response times (IRTs), which represent the period between lever presses, were also examined for the DRL task. IRTs were divided into 2.5-s intervals (e.g., 0–2.5 s, 2.5 – 5.0 s, etc.) with any IRTs longer than 17.5-s falling in the last IRT interval. The IRT data from the first block (initial testing block) and the last block (final testing block) were analyzed separately using a 3 (dose) $\times$ 3 (age) $\times$ 2 (cohort) $\times$ 8 (IRT interval) mixed ANOVA where IRT interval (1–8) was a repeated measures factor.

2.9.3 Reversal Learning Task—For spatial reversal learning, the cumulative number of errors to criterion served as the measure of overall learning rate. The number of errors was calculated by summing the number of errors across all sessions within original learning or a reversal. The data were analyzed using a 3 (dose) $\times$ 3 (age) $\times$ 2 (cohort) $\times$ 6 (original learning + 5 reversals) mixed ANOVA where reversal was a repeated measures factor. Lever press latencies for correct and incorrect presses were also examined.

2.9.4 Dopamine receptor densities—For comparing dopamine receptor expression, the ratios of protein to β -actin were calculated for each rat. Average ratios for dopamine D1, D2 and DAT were analyzed with a two-way between subjects ANOVA for dose and age.

3. Results

3.1 Blood Genistein Concentrations

For serum samples in which total genistein was below the detection limit (0.05 μ M) a serum genistein concentration of 0.00 was assumed for the purposes of statistical analysis (a total of 22 for sucrose control, 1 for low dose, and 0 for high dose); otherwise measured levels were used. The blood genistein levels of the dose groups differed as revealed by a significant main effect of genistein dose, $F(2, 66) = 153.885$, $p < 0.001$. Tukey post hoc analyses found significantly higher serum genistein levels in the high genistein treatment group than in the control group ($p < 0.001$; Table 1). Serum levels in the low dose and high dose groups also differed ($p < 0.01$). However, because of the low total serum levels and relatively large amount of variability in the low dose group, the difference between the low dose group and the control group did not reach statistical significance ($p = 0.149$). The aglycone content typically ranged from 0.5 – 2.0% of total genistein in all treatment groups. Although serum genistein concentrations appeared to decrease with age in the high genistein

dose group, the main effect of age and the age by genistein dose interaction were not significant (Table 1).

3.2 Cohort Effects

Because the experiment was conducted in two replicates or “cohorts” of animals spaced 6 months apart, cohort was included as a factor in all statistical analyses. While some statistical interactions involving cohort were observed, overall there were few effects of cohort. Where cohort effects did occur they tended to be higher order interactions that did not involve treatment group. All age and exposure groups were equally represented in each of the two cohorts and there were no indications that cohort had any significant impact on the study findings. Therefore, in order to simplify this report cohort effects are not discussed further.

3.3 Delayed Spatial Alternation Task

3.3.1 Cued and Non-cued Alternation Training—Genistein treatment did not alter performance on the CA or NCA phases of training for the DSA task, $p > 0.05$. However, age did have a significant impact on NCA training, $F(2, 124) = 12.647$, $p < 0.001$. Post hoc comparisons revealed that young rats performed better than both the middle-aged and old rats on NCA, $p < 0.05$; and middle-aged rats also performed better than old rats, $p < 0.05$. As expected, the proportion correct significantly increased across the 10 sessions of testing ($F(9, 1116) = 195.781$, $p < 0.001$; data not shown).

3.3.2 Proportion Correct on DSA—Genistein treatment impaired DSA performance in old rats relative to both young and middle-aged rats. This effect was revealed by a genistein dose $\times$ age $\times$ testing block interaction for proportion correct, $F(16, 496) = 2.107$, $p = 0.046$ (Figure 1). Post hoc tests for simple interactions (block $\times$ age at each genistein dose) found a significant block $\times$ age interaction for the high genistein dose only, $F(8, 188) = 6.357$, $p = 0.001$. Tukey post hoc analyses found high dose genistein old rats to perform more poorly than either the young and middle-aged high genistein rats in block 5 only, $p < 0.05$ (Figure 1C). The block $\times$ genistein dose interactions at each age were not significant, $p > 0.05$.

Overall, the performance of old rats on the DSA task was impaired relative to that of young and middle-aged rats as revealed by a significant age $\times$ block $\times$ delay interaction, $F(32, 1984) = 2.647$, $p = 0.001$ (Figure 2). Moreover, rats at all ages performed progressively worse with increasing delay with age differences diminishing as delay increased. The simple block $\times$ delay interactions were significant for all age groups, $p < 0.05$. All age groups improved across blocks of testing at the 0, 3, 6 and 9 second delays, but not at the 18-second delay (Figure 2). The 18-second delay exceeded the difficulty threshold for rats on this task, as illustrated by percent correct performance near chance levels in all groups (Figure 2E). Tukey post hoc analyses at each block for each delay found that at the 0 second delay the old rats performed worse than both the young and middle-aged rats at all 5 blocks of testing, $p < 0.05$ (Figure 2A). At the 3-second delay, old rats performed worse on blocks 3–5 of testing $p < 0.05$ (Figure 2B). At the 6-second delay old rats performed worse than young rats during block 4 and worse than both young and middle-aged rats during block 5 of testing (Figure 2C). At the 9-second delay, old rats performed worse than the young rats during block 5, $p < 0.05$ (Figure 2D). Interestingly, at both the 6 and 9-second delays, the old rats performed better than both young and middle-aged rats during the first block of testing $p < 0.05$ (Figures 2C and 2D). There were no differences between groups at the 18-second delay (Figure 2E).

A significant block $\times$ delay effect was also uncovered, $F(16, 1984)=53.004, p<0.001$. As expected, performance improved across blocks of testing, but this pattern became less pronounced as the delays became longer (Figure 2A–E).

3.3.3 Win-Stay and Lose-Stay Errors on DSA—Genistein treatment increased the number of win-stay errors in the high dose old rats relative to high dose young and middle-aged rats. This was revealed by a significant genistein dose $\times$ age $\times$ block effect on win-stay errors, $F(16, 496)=2.311, p=0.018$. Subsequent tests for simple age $\times$ block interactions at each dose found a significant block $\times$ age interaction for the high dose genistein group only, $F(8, 188)=3.042, p=0.016$ (Figure 3). Consistent with their deficit in percent correct during the 5th block of testing (Figure 1c), Tukey post hoc analyses found the high dose genistein old rats to commit more win-stay errors in the 5th block of testing than young, but not middle-aged, high dose genistein rats, $p<0.05$.

A marginally significant main effect of age was observed for win-stay errors, $F(2, 124)=2.653, p=0.074$. The old rats tended to make more win-stay errors, on average, than the young rats did (data not shown). As expected, win-stay errors decreased across blocks of testing as revealed by a main effect of block $F(4, 496)=267.548, p<0.001$. The block $\times$ dose interactions at each age were not significant, $p>0.05$.

Repeated measures ANOVA failed to find any effects of genistein dose on lose-stay errors. A significant block $\times$ age interaction was uncovered, $F(8, 496)=2.238, p=0.049$ (data not shown). Although subsequent comparisons at each block of testing failed to show significant differences, old rats (Mean $\pm$ SEM: 22.91 ± 0.92) tended to make more lose-stay errors than did younger rats (21.63 ± 0.80). A main effect of block was also found, $F(4, 496)=370.086, p<0.001$. As expected, lose-stay errors decreased across blocks of testing.

3.3.4 Lever Press Latencies on DSA—Lever press latencies following either a correct or an incorrect response were shorter in the young rats ($p<0.05$). On average, regardless of treatment group, young rats had a shorter latency than both middle-aged and old rats through all blocks of testing. Genistein dosing, overall, did not impact lever press latencies. Furthermore, as expected, lever press latencies after either a correct or an incorrect response decreased across blocks of testing ($p<0.05$).

3.4 Differential Reinforcement for Low Rates of Responding

3.4.1 Total Presses—Repeated measures ANOVA failed to find any effects of genistein dose on the total number of lever presses per session. However, a significant main effect of age was found, $F(2, 122)=3.380, p=0.037$. Tukey post hoc analyses found the young rats to make significantly more presses than the middle-aged rats, $p<0.05$ (Figure 4). A similar increase in responding was seen in comparison to old rats, but this effect did not reach significance, $p>0.05$. The number of lever presses decreased significantly across blocks of testing for all rats $F(5, 610)=124.349, p<0.001$.

3.4.2 Ratio of Reinforced:Nonreinforced Lever Presses—There were no dose $\times$ block or age $\times$ block effects measured on the ratio of reinforced to non-reinforced lever presses. However, a marginally significant main effect of genistein dose was found on the ratio of reinforced to non-reinforced lever presses, $F(2, 122)=2.979, p=0.055$. The control group tended to have a higher ratio of reinforced responses in comparison to the high genistein group (Figure 5). As expected, the ratio of reinforced:nonreinforced responses increased across blocks of testing, as revealed by a significant main effect of block, $F(5, 610)=168.764, p<0.001$.

3.4.3 Inter-response Times

3.4.3.1 Initial Testing Block: In the first block of training, the control group made more responses than the genistein groups in the response bins associated with reward (15–17.5 sec and > 17 sec), although the dose $\times$ IRT bin interaction did not quite reach statistical significance, $F(14, 854)=2.089$, $p=0.076$ (Figure 6A). An age $\times$ IRT bin interaction was also present during the initial phase of testing, $F(14, 854)=2.648$, $p=0.029$. Tukey post hoc analyses found the middle-aged rats to make more lever presses in the 7.5–10.0 second IRT bin than did young rats, and old rats to make more lever presses than young rats in the >17.5 second IRT bin, $p<0.05$ (Figure 6B). A significant main effect of IRT bin was also present, $F(7, 854)=137.133$, $p<0.001$. The number of responses in each IRT bin tended to decrease as the IRT became longer.

3.4.3.2 Final Testing Block: A marginally significant dose $\times$ IRT bin interaction was also present in the last block of testing, $F(14, 854)=2.264$, $p=0.053$. The control group tended to make fewer responses than the high dose genistein rats in the first IRT bin, while making more responses than both genistein groups in the last IRT bin (Figure 7A). A significant age $\times$ IRT bin interaction was also present in the final block of testing, $F(14, 854)=5.698$, $p<0.001$. Tukey post hoc analyses found that compared to the middle-aged and old rats, the young rats made more responses in the <2.5 second bin, while making fewer responses than both the middle-aged and old rats in the 10.0–12.5 second bin, $p<0.05$ (Figure 7B). Young rats also made fewer responses than did the middle-aged rats in the 2.5–5.0 and 7.5–10.0 bins, $p<0.05$ (Figure 7B). A significant main effect of IRT bin was also found, $F(7, 854)=170.030$, $p<0.001$. As with the initial testing phase, the number of responses in each bin tended to increase as the IRT became longer.

3.5 Reversal Learning: Errors and Latencies

Repeated measures ANOVA failed to find any significant effects of genistein treatment or age at testing on the reversal learning task, however, as expected, all rats made substantial errors on the first reversal and fewer errors in subsequent reversals (main effect of reversal $F(5, 595)=135.971$, $p<0.001$, Figures 8A and 8B). Similar to the DSA task, a significant effect of age was found for lever press latencies following either a correct, $F(2, 119)=12.123$, $p<0.001$, and an incorrect response, $F(2, 119)=10.446$, $p<0.001$. Young rats had shorter lever press latencies following either correct or incorrect responses than both middle-aged and old rats, $p<0.05$ (data not shown).

3.6 Receptor Analyses

Both genistein exposure and age significantly impacted PFC DAT expression, whereas no changes in PFC D1 or D2 dopamine receptors were observed. Statistical analysis revealed a significant dose $\times$ age interaction for DAT expression, $F(4, 47)=3.908$, $p=0.008$, and follow up tests of simple main effects at each dose revealed a significant effect of dose in the old age group, $F(2, 12)=13.024$, $p=0.001$. Tukey post hoc analyses revealed significant differences between the control group and both the low and high genistein dose groups, $p<0.05$, with the genistein groups showing a higher ratio of DAT: β -actin (Table 2). Tests of simple main effect also revealed a significant effect of age in the control group, $F(2, 17)=5.834$, $p=0.012$. Tukey post hoc analyses revealed a significant difference between old and middle aged rats, $p<0.05$, with old rats showing a lower ratio of DAT: β -actin (Table 2).

4. Discussion

The current study examined the effects of chronic dietary genistein exposure in young, middle-aged and old ovariectomized female rats on a cognitive battery which included tests of working memory (DSA), inhibitory control and timing (DRL), and strategy shifting

(spatial RL). Overall, effects of dietary genistein were seen on the DSA and DRL tasks, impairing the performance of the old rats on the DSA task, while producing an overall trend towards inefficiency in responding in all age groups on the DRL task. Age also impacted performance as the old rats tended to perform worse on the DSA task overall, while middle-aged and old rats tended to perform better than young rats on the DRL task. The reversal learning task failed to show effects of genistein treatment or age on errors committed, but did find an impact of age on lever press latencies. We also observed a decrease in DAT expression in old rats relative to middle-aged rats, and a genistein-induced increase in the density of DAT in the PFC of genistein-treated old rats relative to similarly aged controls.

It is important to note that the young rats used in this study were nulliparous whereas the middle-aged and old rats were retired breeders. Studies have linked reproductive experience to improved cognitive function, but these effects are often transient in nature or seen in rats that have given birth to a single litter [63,64]. Although the reproductive histories of the young rats which were nulliparous and middle-aged rats which were retired breeders differed, their performance was similar on the DSA task. In contrast the performance of both the young and middle-aged rats differed from that of the old rats which were also retired breeders. Similar effects were also seen on the DRL task. Therefore, the effects measured here do not appear to be related to reproductive history.

4.1 Genistein and Executive Function

Chronic treatment with the higher dose of genistein produced a decrement in DSA performance of the old rats relative to both the high dose young and middle-aged genistein groups during the last block of testing, an effect likely due to the fact that the old high genistein rats committed more win-stay errors during that block. As Figure 1 illustrates, the performance of old high dose genistein treated rats was relatively flat across the 5 blocks of testing, whereas the control and low genistein groups improved across testing blocks. On the DRL task, there was also a trend for high genistein treatment to decrease the ratio of reinforced to non-reinforced lever presses relative to the control group. Genistein treated rats tended to make more responses in the shortest IRT bins, an effect similar to what has been seen in estradiol treated rats in another task of timing estimation [74]. In contrast, no evidence of an effect of genistein treatment on reversal learning was observed. The pattern of effects in genistein-treated rats was similar to, although more subtle than, that in estradiol treated rats [90,91], with some evidence of impaired performance on both the DSA and DRL tasks, but no evidence of impairments on the reversal learning task. This is consistent with effects in non-human primates, for which performance on reversal learning tasks is relatively insensitive to estrogen treatment [38,39,89].

Our current results stand in contrast to previous rodent work that found chronic soy supplementation in adult ovariectomized rats produced improvements in cognitive function [47,53,62,75]. It is important to note that these previous studies used tasks that have been shown to engage the hippocampus, with the effects of soy being similar to what is generally observed with estradiol treatment on the same tasks [15,20]. Long thought to prevent age-associated cognitive decline, estrogen treatment has recently been shown to have negative effects on some types of cognitive tasks, particularly those that tap brain regions outside the hippocampus [e.g. 31,90,91,92]. Our current results suggest soy phytoestrogens may also have task-specific effects on memory, impairing performance on PFC-mediated tasks of working memory and response inhibition, similar to what we have seen with estradiol.

It is important to note that the genistein doses used in this study were moderate and dosing was conducted only once daily. Blood concentrations achieved one hour after treatment in the high genistein dose group were similar to blood concentrations in humans consuming typical Asian high soy diets [1]. The low genistein dose group concentrations were

comparable to blood concentrations achieved in humans taking some of the soy isoflavone supplements that are currently on the market [14], although due to the disparate amount of genistein content in available soy supplements, the exact levels humans are exposed to vary greatly. As the ~4-hr half life of genistein in the blood of female rats is short [10], the once daily treatment used here would fail to produce constantly elevated genistein concentrations. Additional studies using higher doses of genistein and/or several doses/day are needed to understand more fully the potential for genistein effects on cognition.

4.2 Age and Executive Function

Old rats showed deficits relative to both young and middle-aged rats on the DSA task, especially at shorter delays and in the later blocks of testing, tending to commit more win-stay and lose-stay errors. In contrast, middle-aged and old rats also made fewer lever presses than did young rats on DRL, a task where lower response rates are generally associated with better performance.

Age-related deficits in working memory have been reported in other rodent studies as well as in non-human primates and humans [69,72,79], including both operant and non-operant delayed alternation tasks [52,70,71]. Here, we found 22-month old rats to have a deficit in DSA performance in comparison to both young (7-month) and middle-aged (16-month) rats. Age effects on working memory in the operant DSA task do not emerge until very late in the life span as we failed to measure age-related deficits in 18-month old rats in a previous study [91]. Our current findings corroborate previous research that found operant delayed alternation performance deficits in rats aged 21 months and older [70,71].

In contrast to working memory tasks on which age-related deficits in performance have been demonstrated, few studies have addressed the impact of aging on tasks such as DRL that require inhibitory control. Other researchers have reported that, relative to younger rats, aged rats (>23 months) have lower DRL response rates [42,81]. These data suggest that on the DRL task, where animals are required to wait for a period of time between responses, factors associated with aging, such as decreased impulsivity and decreased overall activity [23], may give older animals a slight advantage over younger animals. Decreased response rates would be expected to bias old rats towards performing better in a task where high response rates correlate with shorter inter-response times and lower efficiency. In keeping with this, old rats in the current study had fewer responses in the shortest IRT bin and more responses with longer inter-response times, a pattern of responding that is conducive to better performance on the DRL task.

There were no significant age differences between groups on errors committed in the reversal learning task, although a significant effect of age was found for lever press latencies, which was similar to the effect measured on the DSA task. Several previous studies in non-human primates have also failed to uncover age associated deficits on reversal tasks [25,37,67,88].

4.3 Dopamine Receptors and DAT

We measured several changes in the expression of DAT modulated by both genistein treatment and aging, while no changes in the expression of the D1 or D2 dopamine receptors in the PFC were found. The PFC is a primary brain area involved in response inhibition and timing [35,86] as well as working memory [3,7,11,51,52]. Dopamine disruption in the medial PFC has been found to impair both response inhibition/timing ability [82] and working memory [5,6]. Therefore, altered dopamine function in the PFC is a potential mechanism underlying the effects of genistein treatment and age observed in this study.

We found a decrease in DAT expression in old control rats relative to middle-aged control rats. Several other studies have found an age induced decrease in DAT expression in brain regions outside the PFC across species [24,28,73,87]. Age-related decreases in basal levels of dopamine and its metabolites in the PFC of aged rats and monkeys have also been reported [22,45]. As the DSA task is sensitive to dopaminergic disruption, age-induced decreases in basal dopamine levels could account for the performance deficit measured in old rats on the DSA task. This hypothesis is supported by recent findings of an age-associated deficit in a T-maze alternation task in 24-month old rats that was associated with reduced dopaminergic transmission in the PFC [52]. DAT expression was also marginally lower in young control rats as compared to middle-aged control rats, although this difference did not reach statistical significance. Typically, young and middle-aged rats have similar expression of DAT in brain regions outside of the PFC [73]. As the effects of ovariectomy on DAT expression during aging in the rodent PFC has not been previously studied, future research will be needed in order to confirm these results.

We also found chronic genistein treatment to increase DAT expression in the PFC of ovariectomized aged rats relative to similarly aged control treated rats. Other researchers have also reported the PFC to be sensitive to the effects of dietary soy [47,60,61]. Several studies have measured an ovariectomy-induced decrease in brain DAT expression in brain regions outside of the PFC, with a subsequent 'restoration' of expression following estradiol treatment [2,54,55]. Similar effects have also been seen with the ER β selective agonist 2,3-bis(4-hydroxyphenyl) propionitrile (DPN) in the striatum [43]. The effects we measured on DAT expression following genistein dosing in aged rats are similar to what is seen with both estradiol and the ER β agonist DPN on DAT expression in brain regions outside of the PFC. Importantly, aging is associated with a decrease in basal levels of dopamine in the PFC of intact rats [45]. As the aged rats used here were ovariectomized, it is difficult to determine how this model would affect the measured loss of PFC dopamine during aging. Even so, with an increase in DAT expression following genistein dosing, underlying changes in DA neurotransmission may account for the performance deficits seen on the DSA task in aged rats receiving the higher dose of dietary genistein. Future research will determine the effects of ovariectomy on dopamine levels in the PFC.

4.4 Conclusion

In summary, this study assessed executive function in ovariectomized young, middle-aged and old rat treated with daily doses of genistein that resulted in blood concentrations similar to those found in humans consuming high soy diets. Three tests were employed to assess distinct aspects of executive functioning including working memory (DSA), inhibitory control (DRL) and cognitive flexibility (reversal learning). Subtle effects of genistein exposure were observed on both the DSA and DRL tasks, but not on the reversal learning task. Although not as striking, the pattern of effects in genistein-treated rats was similar to that of estradiol showing some evidence of impaired performance on both the DSA and DRL tasks [90,91], but no evidence of impairments on the reversal learning task. Age-related effects were apparent on both the DSA and DRL tasks, but were only seen on lever press latencies on the reversal learning task. Old rats performed more poorly than young and middle-aged rats on the DSA task, but had lower overall response rates on the DRL task, an indication of better performance. DAT expression in the PFC was also found to be decreased in control old rats, while genistein treatment increased DAT levels in old rats only. These results highlight the fact that specific aspects of executive function are sensitive to both dietary estrogen exposure and aging. Future studies employing higher doses of genistein that model the higher blood concentrations achievable in humans taking high dose soy isoflavone-containing dietary supplements will be important to determine whether more striking cognitive deficits occur at higher levels of genistein exposure.

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Figure 1.

Proportion correct on the DSA task across 5-day blocks of testing for (A) control groups, (B) low dose genistein groups and (C) high dose genistein groups. The high dose old genistein group performed significantly worse than either the high dose young or middle-aged genistein groups on the last block (* $p < 0.05$).

Figure 2. Proportion correct on the DSA task across blocks for different delays in young, middle-aged and old rats: (A) 0 second delay; (B) 3 second delay; (C) 6 second delay; (D) 9 second delay; and (E) 18 second delay; * indicates a significant difference between old and both young and middle aged groups ($p < 0.05$); ** indicates a significant difference between old and young age groups ($p < 0.05$).

Figure 3.

Total win-stay errors committed on the DSA task across 5-day blocks of testing by the high dose genistein groups. The high dose old genistein group committed significantly more win-stay errors than the high dose young genistein group (* $p<0.05$).

Figure 4. Total lever presses at each age averaged across all 30 test sessions during DRL. Young rats made significantly more lever presses than middle-aged rats (* $p < 0.05$).

Figure 5.

Ratio of reinforced:non-reinforced lever presses at each dose group averaged across all 30 test sessions on the DRL task. The control groups had a nearly significant increase in ratio of reinforced:nonreinforced lever presses than the high dose genistein groups. +indicates a near significant main effect of genistein dose group ($p=0.055$) with a near significant difference between high dose genistein and control groups ($p=0.098$).

Figure 6.

(A) Proportion of total responses in each 2.5 sec interresponse time (IRT) bin during the initial training block for genistein dose groups. (B) Proportion of total responses in each 2.5 sec interresponse time (IRT) bin during the initial training block for age. Young rats made significantly fewer responses in the 7.5–10.0 sec IRT bin than middle-aged rats (* $p < 0.05$), also making significantly fewer responses than old rats in the <17.5 sec IRT bin (** $p < 0.05$).

Figure 7.

(A) Proportion of total responses for each 2.5 sec interresponse time (IRT) bin during the final testing block for genistein dose groups. (B) Proportion of total responses for each 2.5 sec interresponse time (IRT) bin during the final testing block for age. Young rats made significantly more responses in the <2.5 sec IRT bins than both the middle-aged and old rats ($*p<0.05$), while making significantly fewer responses in the 10.0–12.5 sec IRT bin than both middle-aged and old rats ($*p<0.05$). Young rats also made significantly fewer responses in the 5.0–7.5 sec and 7.5–10.0 sec IRT response bins than the middle-aged group ($**p<0.05$).

Figure 8.

(A) Ratio of dopamine transporter (DAT) to β -actin for the old age group across genistein doses. The old control treated group had a significantly lower ratio of DAT: β -actin than both the low and high dose old genistein groups ($*p < 0.05$). (B) Ratio of dopamine transporter (DAT) to β -actin for control groups across age. The old control group had a significantly lower ratio of DAT: β -actin than the middle-aged group ($*p < 0.05$).

Table 1

Serum levels of total genistein collected at completion of behavioral testing battery, 1 hour after single dose. Values are in mean $\pm$ SEM μ M (n).

	Young	Middle Age	Old
Control	<LOD (8)	0.23 $\pm$ 0.19 (10)	0.33 $\pm$ 0.33(7)
Low Dose	0.76 $\pm$ 0.19 (10)	0.73 $\pm$ 0.31 (8)	0.79 $\pm$ 0.37 (7)
High Dose	2.47 $\pm$ 0.62(10)	1.83 $\pm$ 0.43(10)	1.15 $\pm$ 0.45(5)

Table 2

DAT:βactin expression in the prefrontal cortex at the completion of behavioral testing battery.

	Young	Middle Age	Old	Mean
Sucrose	0.59 ± 0.05 (9)	0.79 ± 0.08 (7)	0.46 ± 0.01 (4) ^{a,b}	0.63 ± 0.04 (20)
Low Dose	0.70 ± 0.07 (8)	0.70 ± 0.06 (7)	0.76 ± 0.02 (6)	0.72 ± 0.03 (21)
High Dose	0.68 ± 0.07 (6)	0.54 ± 0.08 (4)	0.74 ± 0.07 (5)	0.66 ± 0.05 (15)
Mean	0.65 ± 0.04 (23)	0.70 ± 0.05 (18)	0.67 ± 0.04 (15)	

^aThe sucrose treated old group had a lower DAT:βactin ration than either the low or high dose old groups ($p < 0.05$).

^bThe old sucrose treated rats also has a lower DAT:βactin ration than the middle-aged sucrose group ($p < 0.05$).

Values are in mean ± SEM DAT: βactin ratio (n).