Dietary phytoestrogens enhance spatial memory and spine density in the hippocampus and prefrontal cortex of ovariectomized rats

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A number of recent studies show that phytoestrogens exert physiological effects in rats and humans (see Lephart et al. (2002) and Lephart et al. (2005) for review). Phytoestrogens are similar in structure to estradiol and are found in some vegetables, whole grains, soy and clover products (Patisol, 2005). Depending on the physiological state of the subject and the specific phytoestrogen, these compounds may act as estrogenic agonists or antagonists, but their potency is much lower than estradiol (MacLusky, 1997). Animal feeds, including rodent chows, generally derive protein from alfalfa and soybean meal which are rich in isoflavones and coumestans, two of the more potent phytoestrogens (Lephart et al., 2002). Thus, it is possible that dietary phytoestrogens might influence estrogen-sensitive neural and physiological systems. Indeed, Lephart and colleagues and others have compared rats receiving high phytoestrogen chow and phytoestrogen-free chow and found some differences in brain morphology and behavior (Lephart et al., 2002, 2005; Patisol, 2005; Perez-Martin et al., 2005).

In this study, we compared ovariectomized (OVX) rats that consumed the widely used, standard rodent chow, Purina LabDiet® 5001 with those that consumed Harlan 2016 Teklad Global 16% Protein Rodent Diet chow. Purina LabDiet chow contains approximately 810 μg/g of phytoestrogens, derived mainly from soymeal (Brown and Setchell, 2001) while the 2016 Teklad chow contains no soy or alfalfa, and therefore contains minimal phytoestrogens, but is not phytoestrogen free (see http://www.teklab.com). We investigated whether behavioral, physiological and neural properties, which are known to be influenced by estradiol, would be affected in OVX rats that chronically ingested diets differing in phytoestrogen levels. Levels of dietary phytoestrogens might be an important factor to consider in experiments which investigate long-term effects of estrogens.

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OVX, 2-month-old Sprague-Dawley rats were purchased from Harlan Sprague Dawley, Inc., housed in pairs on a 12:12-h light-dark cycle (lights on at 7 am) and given food and water ad libitum. All experimental procedures were approved by the Hunter College Institutional Animal Care and Use Committee. Upon arrival, they were randomly divided to receive our standard Purina LabDiet® 5001 (designated as High Phyto Chow, n=8) or Harlan 2016 Teklad Global 16% Protein Rodent Diet (designated as Low Phyto Chow, n=9).

Following 1 week of acclimation, subjects received 2 weeks of habituation to the memory tasks which consisted of open field and object recognition/placement trials with consecutively longer inter-trial delays (see below). Behavioral tests were conducted between weeks 5–9 (see text), and rats were sacrificed at the end of the 9th week. Object recognition and placement trials were conducted as previously reported by the laboratory (Beck and Luine, 1999; Luine et al., 2003) and given in alternate weeks to preclude order effects. Briefly, object recognition trials consisted of a sample trial (T1) and a recognition trial (T2). The two trials were separated by an inter-trial interval of varying lengths. In T1, two identical objects were placed at one end of the open field and amount of time spent exploring the two objects was recorded for 3 min. For T2, or the recognition trial, a new object replaced one of the previous objects. In T2, the time spent exploring the old (familiar object) and the new (novel) object was recorded for 3 min. Exploration was defined as when the subject sniffed at, whisked at, or looked at the object from no more than 2 cm away. The objects used for trials were various bottles, cans, and containers, and the novel object was counterbalanced across treatments. The field and objects were cleaned after each individual trial. All animals received pre-testing acclimation sessions with inter-trial delays of 1 min, 10 min, 1 h, 2 h and 4 h. Then spatial memory acclimation trials were given using the object placement (OP) task, a spatial memory task known to be hippocampus-dependent (Beck and Luine, 1999; Luine et al., 2003). This task is identical to the OR procedure, except that during T2, the recognition trial, one object is moved to a novel location within the field (instead of replacing one object with a new object). Time spent by each subject exploring objects at the old and new location was recorded during T2. The objects consisted of various small candlesticks and statues. All animals received 10-min, 1-h and 2-h acclimation trials. The new location was counterbalanced across groups and treatments. All subjects explored the objects during both T1 and T2, and no differences between the groups in exploration times during T1 were found (data not shown). A 4-h inter-trial delay was used to test performance in both tasks (Fig. 1).

Anxiety levels were measured using the elevated plus maze (Lund et al., 2005). Time spent on the open and closed arms during a 6-min trial was recorded. Entry into an arm was noted when the subject placed all four limbs in the arm. Subjects were sacrificed via rapid decapitation at week 9 after initiation of the diets, and brains and uteri were removed. Brains were placed in solutions obtained from FD NeuroTechnologies and uteri weighed.

Golgi impregnation was used to quantify the number of dendritic spines in the hippocampus and prefrontal cortex of subjects. The FD Rapid Golgistain™ Kit and associated methodology was utilized (FD NeuroTechnologies Consulting and Services, Inc.). Brains were rinsed in 0.1M phosphate buffer, immersed in Golgi-Cox solution for 14 days and then transferred to a sucrose containing solution at 4 °C for 2–7 days. The hippocampus and prefrontal cortex were sectioned at 100 µm and apposed to gelatin-coated slides with a drop of sucrose solution. Slides were left to air dry at room temperature for 1 week in the dark, then processed for Golgi impregnation as outlined in the FD Rapid Golgistain™ Kit.

Dendritic spine density of pyramidal neurons in the prefrontal cortex (layer II/III) and hippocampus (subfield CA1) was analyzed using the Spot Advanced program, version 3.5.5 for Windows (©Diagnostic Instruments, Inc., 1997–2002) and a Nikon Eclipse E400 microscope. Pyramidal neurons were located and spines were counted according to the general methods of Gould et al. (1990) in the hippocampus and Robinson and Kolb (1999) in the prefrontal cortex. Dendrites had to meet several criteria to be included: first, cell bodies needed to be within the area of interest; second, the length of the branch was unbroken, and third, the length of the dendrite was isolated well enough for an unobstructed view. These criteria were applied to ensure that the same population of dendrites and spines were analyzed within and across subjects. At least 5–6 tertiary dendrites meeting these criteria from CA1 and prefrontal cortex of each subject were measured for length using the Spot Advanced software. Spines on the branches were counted under oil, and the spine density/µm of the dendrites was calculated and averaged for each subject. Data were analyzed using a two-way ANOVA (area vs. diet).

Results of object recognition and placement testing during weeks 5–9 of feeding are shown in Fig. 1. For object placement, the exploration ratio (%time at new location) was determined at weeks 5 and 7 on the diets (Fig. 1A). Data were analyzed by two-way repeated measures ANOVA (group vs. week, with week as repeated measure). No main effects were found, but a significant interaction effect was present ($F_{1,28}=4.71, p<0.04$). Post hoc testing by Mann–Whitney U test showed that ratio in the High Phyto Chow group at week 7 was significantly different from the Low Phyto Chow group ($Z=2.11, p<0.03$) with exploration ratios of 68 vs. 50%, respectively. Object recognition performance was tested at weeks 6 and 8 following initiation of the diets (Fig. 1B); since no differences between groups were noted, an additional test was given at week 9. Analysis by two-way repeated measures ANOVA of the three trials of object recognition testing (group vs. week, with week as repeated measure) showed no significant main or interaction effects. Thus, object placement performance was affected by the content of phytoestrogens in the diet at 7 weeks after initiating feeding whereas object recognition performance was not altered up to 9 weeks following initiation of the phytoestrogen diets.

Elevated plus maze performance was tested during week 9 on the diets. No group differences in time spent in the open or closed arms were found (Fig. 1C). In addition, the number of entries was not different between the high and low Phyto chow groups (open arm entries: High Phyto=$0.88±0.39$, Low Phyto=$1±0.46$; closed arm entries: High Phyto=$8±4.5$, Low Phyto=$6.1±0.97$).

At sacrifice (post 9 weeks on the diets), the uteri from the low phytoestrogen chow group weighed 15% less than the
high phytoestrogen chow group (Fig. 2A). Body weight did not differ between the groups (data not shown). The density of spines on tertiary dendrites of pyramidal neurons in the CA1 region of the hippocampus and prefrontal cortex were measured and analyzed by two-way ANOVA (area×diet). Significant effects of area (F1,24=15.4, p<0.001) and diet (F1,24=7.8, p<0.01) were found. Thus, higher spine density was present in CA1 pyramidal neurons than in prefrontal cortex layer II/III pyramidal neurons. The low phytoestrogen diet also resulted in lowered spine densities in both CA1 and prefrontal cortex tertiary apical dendrites. Neurons in CA1 and prefrontal cortex of the Low Phyto Chow group had, respectively, 68% and 79% of the spine density present in the High Phyto Chow diet group.

These results show that 7–9 weeks following the initiation of feeding rodent chow differing in phytoestrogen content, OVX adult rats show differences in some behavioral and neural parameters. Specifically, spatial memory performance using the object placement test, dendritic spine densities and uterine weights were higher in rats fed the Purina LabDiet® 5001 chow which is high in Phytoestrogens than in the Teklad Global 2016 Rodent Diet which is low in phytoestrogens. The particular phytoestrogens responsible for the changes are unknown, but the Teklad Global 2016 does not contain alfalfa or soybean meals as does Purina LabDiet® 5001 chow (http://www.teklad.com, rodent diet data). A variety of phytoestrogens are present in alfalfa and soya bean meal, some of which have a greater affinity for estrogen receptor α than β and vice versa (Patisol, 2005). Since the uterus contains only α-estrogen receptors (Hewitt and Korach, 2003), and uteri from rats fed the high phytoestrogen chow were heavier than those fed the low phytoestrogen chow, phytoestrogens active at α-estrogen receptors are present in the Purina chow.

The brain contains both α and β estrogen receptors (Shughrue et al., 1997), and the hippocampus and prefrontal cortex contain moderate to high levels of both receptors (McEwen, 2002; Mitra et al., 2003). Phytoestrogens, like estrogens, have been shown to enter the brain rapidly and to bind estrogen receptors in the frontal cortex and hippocampus (Lephart et al., 2002; Lund et al., 2001). However, the
binding affinities of phytoestrogens are much lower than estradiol (see Patisol. 2005 for further discussion). In relation to mediation of memory, object placement is dependent on an intact hippocampus and/or fornix (Ennaceur et al., 1997), and may also rely on prefrontal cortical input (Ennaceur and Aggleton, 1994). Object recognition, however, is less dependent on the hippocampus and requires prefrontal cortical input (Broadbent et al., 2004; Ennaceur and Aggleton, 1994). Lesions of at least 80% of the hippocampus are necessary to impair object recognition while smaller lesions of the hippocampus (30%) impair spatial memory tasks (Broadbent et al., 2004). On the other hand, performance of both tasks is enhanced by estrogen treatment to O VX rats (Li et al., 2004; Luine et al., 2003) and is impaired in O VX rats (Luine et al., 2003; Wallace et al., 2006). Thus it was surprising that object placement performance was significantly lower in rats fed the diet low in phytoestrogens as compared to the diet high in phytoestrogens at 7 weeks after initiation of the diets but object recognition performance was not different in the groups at the intervals tested, 6, 8 and 9 weeks after initiation of the diets.

Estrogen administration to O VX rodents or monkeys increases dendritic spine density or spine synapse density in CA1 (Gould et al., 1990; Leranth et al., 2002; Li et al., 2004; MacLusky et al., 2005) and ovariectomy decreases CA1 dendritic spine density (Gould et al., 1990; Wallace et al., 2006). Doses of estradiol which increase spine density are associated with better performance of object placement (Li et al., 2004) and Morris water maze (Sandstrom and Williams, 2001). Thus, it is possible that the 48% increase in spine density in CA1 of the rats fed the high phytoestrogen diet as compared to the low phytoestrogen diet is responsible for the enhancement in object placement performance. O VX is also associated with decreased object recognition performance (Luine et al., 2003; Wallace et al., 2006) and estrogens given to O VX rats enhance performance (Luine et al., 2003). O VX is also associated with decreased prefrontal cortex spine density (Wallace et al., 2006), and in Rhesus monkeys, estradiol replacement increases Spinophilin-immunoreactive spine number in this area (Tang et al., 2004); however, effects of estrogens on spine density in this region have not been examined in rats. Spine density in high Phyto diet rats was only 25% higher than the low Phyto subjects in the prefrontal cortex. The smaller difference in prefrontal cortex spine density between the groups, as compared to 48% higher spine densities in CA1 in high Phyto diet group, may underlie the lack of difference in object recognition performance between these groups. Since performance of both memory tasks in the low Phyto diet group appears to be decreasing over time, a longer interval on the low Phyto diet may be associated with a further reduction in frontal cortex spine density and significantly decreased object recognition performance.

Based on the current data, it is unclear whether a difference in the type of estrogen receptors present in brain areas might be responsible for the differences in behavioral and neural responsivity to the phytoestrogen diets or whether differences in density of estrogen receptors between the brains areas might be responsible for the differences in the cognitive responses. Soy extract also increases the production of new cells in the dentate gyrus area of the hippocampus in old female rats (Perez-Martin et al., 2005), and changes in phytoestrogen content of the diet influence the size of the anteroventral periventricular nucleus of females (Lephart et al., 2005). Thus, differences in types of phytoestrogens as well as phytoestrogen-dependent changes in a number of brain areas may have influenced the diverse behavioral responses.

Previous studies have shown effects of phytoestrogens in the diet on brain morphology and behavior, but these studies were conducted under different treatment paradigms than this study. Gonadally intact female rats were given lifelong exposure to a phytoestrogen-free diet or one with high dietary soy-derived phytoestrogens. Subjects on the high dietary soy-derived phytoestrogen diet showed

Fig. 2 – (A) Uterine Wet Weight. Uterine wet weight (grams) is shown for rats receiving Purina LabDiet® 5001 chow which contains high phytoestrogen levels (High Phyto Chow, open bars) or Harlan 2016 Teklad Global chow which contains low amounts of phytoestrogens (Low Phyto Chow, solid bars). Entries are the average ± SEM for determinations in 8 subjects in Chow and 9 subjects in Low Phyto Chow. Weights determined 9 weeks after initiating the diets. Differences tested by t-test, *p<0.05. (B) Dendritic Spine Density. Dendritic spine densities (spines/μm) are shown for rats receiving Purina LabDiet® 5001 chow which contains high phytoestrogen levels (High Phyto Chow, open bars) or Harlan 2016 Teklad Global chow which contains low amounts of phytoestrogens (Low Phyto Chow, solid bars). Entries are the average ± SEM for determinations in 6 subjects in High Phyto Chow and 6 subjects in Low Phyto Chow. Counts were made 9 weeks after initiating the diets. Data analyzed by two-way ANOVA (area vs diet); significant effects of area (F1,24=15.4, ***p<0.001) and diet (F1,24=7.8, **p<0.01) were found.
better acquisition of the spatial memory task, radial arm maze (Lund et al., 2001), a result similar to the current effect on object placement. When tested for anxiety on the plus maze at 120 days of age, phytoestrogen-rich diet fed rats were less anxious than phytoestrogen-free diet fed subjects (Lephart et al., 2002). In the current study, no effects of phytoestrogen levels in the diet on anxiety were found, but the current treatment regimen was much shorter.

In conclusion, we have found differences in brain morphology and cognitive behavior when OVX females are maintained long term on diets differing in amounts of phytoestrogens. Dietary effects were present from 7–9 weeks following initiation of the diets. While further studies are necessary to determine the compounds responsible for the differences, these data highlight that the type of rat chow provided to ovariectomized rats is sufficient to alter physiological and neural responses in long-term experiments. Thus, in chronic experiments assessing effects of gonadal hormones in OVX rats, phytoestrogens in the diet may exert critical influences on experimental outcomes and should therefore be considered in designing experimental protocols.

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References


