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The effects of the botanical estrogen, isoliquiritigenin on delayed spatial alternation

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ABSTRACT

Age-related declines in cognitive function can impair working memory, reduce speed of processing, and alter attentional resources. In particular, menopausal women may show an acceleration in the rate of cognitive decline as well as an increased vulnerability to brain diseases as estrogens may play a neuroprotective and neurotrophic role in the brain. To treat menopausal symptoms, many women turn to botanical estrogens that are promoted as a safe and natural alternative to traditional hormone replacement therapy. However, the majority of these compounds have not been systematically evaluated for efficacy and safety. The current study investigated the efficacy of the commercially available botanical estrogenic compound isoliquiritigenin (ISL) to alter performance on an operant working memory task, delayed spatial alternation (DSA). ISL is a compound found in licorice root that has been shown to have a wide range of effects on different biological systems, including estrogenic properties. This botanical is currently being used in over the counter dietary supplements. Middle-aged (12-month old) Long-Evans female rats were ovariectomized and orally dosed with either 0 mg, 6 mg, 12 mg or 24 mg of ISL 60 min before testing on the DSA task. The DSA task required the rat to alternate its responses between two retractable levers in order to earn food rewards. Random delays of 0, 3, 6, 9 or 18 s were imposed between opportunities to press. ISL treatment failed to alter DSA performance. Previous work from our research group has found that estrogenic compounds, including 17β-estradiol and the botanical estrogen genistein impair performance on the DSA task. The goal of our botanical estrogens research is to find compounds that offer some of the beneficial effects of estrogen supplementation, without the harmful effects. This work suggests that ISL may not carry the cognitive risks associated with most other estrogenic compounds tested to date.

1. Introduction

Botanical estrogens are non-steroidal plant compounds that can mimic estrogen in the body (Glazier and Bowman, 2001). These compounds are widely sold as dietary supplements despite a dearth of research on their effects (Mahady et al., 2003). Many over the counter botanical supplements contain licorice root powder (Geller and Studee, 2005). Isoliquiritigenin (ISL), and its active metabolite liquiritigenin (LIQ) are two of the primary bioactive compounds in licorice root and both compounds have been demonstrated to have estrogenic activity both *in vivo* and *in vitro* (Maggiolini et al., 2002; Mersereau et al., 2008; Miksicek, 1993).

There is a large body of research indicating that estrogens can affect cognition (Engler-Chiurazzi et al., 2016; McEwen, 2001). However, the effects of estrogens on cognition can vary widely depending on a variety of factors including the cognitive task and the brain areas engaged during completion of the task. Specifically, the prefrontal cortex and the hippocampus are known to play important roles in working memory tasks (D'Esposito, 2007; Floresco et al., 1997). Most rodent tests of working memory have utilized maze paradigms, and these tests often

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involve the use of cue configurations together with relatively long delays between testing trials. Both of these factors likely engage the hippocampus during performance of the task (Maruki et al., 2001; Sloan et al., 2006; Wang and Cai, 2006). In contrast, operant based working memory tasks such as delayed matching to sample or delayed alternation that use short inter-trial delays of < 20 s do not appear to require the hippocampus (Maruki et al., 2001; Sloan et al., 2006). Most work on the effects of estrogens on learning and memory has focused on hippocampus-sensitive tasks, while relatively little work has focused on tasks that rely on the prefrontal cortex. Thus, we have utilized a delayed spatial alternation (DSA) operant task to investigate the effects of estrogens on a prefrontal cortex mediated aspect of learning and memory.

We have previously reported that ovariectomized (OVX) young (3 month old), middle-aged (12 month old) or old (18 month old) rats given physiological levels of 17β-estradiol were impaired on an operant DSA task relative to OVX vehicle control treated rats (Wang et al., 2008; Wang et al., 2009). Wang et al. (2008) tested young (3 month old) OVX rats on the operant DSA task. Rats were implanted with a silastic capsule containing 17β-estradiol (serum level of 20.82 ± 3.41 pg/ml: Wang et al., 2009) or one containing cholesterol. This 17β-estradiol dose was chosen to mimic a high physiological level. We found that chronic 17β-estradiol supplementation resulted in a deficit in DSA performance compared to rats given cholesterol vehicle only. In a follow up study, Wang et al. (2009) tested young (3 month old), middle-aged (12 month old) and old (18 month old) OVX rats on the DSA task. Rats were implanted with a silastic capsule containing 10% 17β-estradiol or cholesterol. We found that chronic 17β-estradiol supplementation resulted in a deficit in DSA performance in all the tested age groups compared to age matched rats given cholesterol vehicle only. In order to further investigate the role of estrogens in modulating performance on the DSA task, middle-aged (12 month old) rats were OVX and given either the ERα agonist propyl pyrazole triol (PPT), the ERβ agonist diarylpropionitrile (DPN) (0.02, 0.08, or 0.20 mg/kg/day for both agonists) or oil control (Neese et al., 2010a). Another group received 10% 17β-estradiol in a silastic capsule to serve as a positive control. This study replicated the estradiol results from Wang et al. (2009), finding that the 17β-estradiol group was impaired on the DSA task compared to age-matched oil control groups. This study also revealed that a low dose of the ERβ agonist DPN impaired performance, but that the ERα agonist PPT did not, suggesting that the 17β-estradiol induced deficit in DSA performance may be ERβ mediated (Neese et al., 2010a).

We have also investigated the effects of the botanical estrogens genistein and S-equol on DSA performance. Genistein is an ERβ selective isoflavone found in soy. S-equol is a metabolite of the soy isoflavone daidzein and is also ERβ selective (Muthyala et al., 2004). Neese et al. (2010b) tested the effects of oral genistein exposure in young (7 month old), middle-aged (16 month old) and old (22 month old) Long-Evans OVX rats on the DSA task. Rats were given genistein orally once daily at either a low dose (162 μg/kg/day) or a higher dose (323 μg/kg/day). We found that the old rats receiving the higher dose of genistein performed worse than both the young and middle-aged groups given that dose. Neese et al. (2012) investigated the effects of three daily exposures to genistein (3.4 mg/kg each time) on DSA and DRL performance in middle-aged (14 month old) OVX rats. This exposure paradigm was designed to keep serum genistein levels in the range of those found in humans consuming commercially available soy isoflavone supplements. We found that there was a trend for genistein treated rats to perform worse than sucrose control rats overall in DSA performance. Genistein treatment did not affect performance on the DRL task. Neese et al. (2014) also tested middle-aged (12–13 month old) OVX rats on the DSA task after treatment with S-equol. S-equol binds selectively to ERβ with an affinity similar to that of genistein but has lower transcriptional potency than genistein (Muthyala et al., 2004). S-equol was given at 10,000 or 19,000 μg/kg/day orally. We found that S-equol did not affect performance on the DSA task.

In the present study, we investigated the effects of ISL on the same operant DSA task used to assess other estrogens in these previous studies. Both ISL and LIQ can bind to estrogen receptors and LIQ has a twenty-fold higher affinity for ERβ over ERα (Mersereau et al., 2008). However, ISL and LIQ readily convert back and forth, and serum levels of the two compounds rapidly come to equilibrium after treatment with either compound (Simmler et al., 2013). Both compounds are lipophilic and have relatively low molecular weight and likely cross into the brain through the blood brain barrier (Srihari et al., 2012).

We chose to treat the rats with ISL, which is more readily available commercially. Similar to other estrogens, we hypothesized that treating OVX middle-aged (12 month old) rats with ISL would result in impairment on the operant DSA task. Dietary supplements containing ISL are already available for consumers to buy with no pre-market regulation. These supplements are marketed primarily to peri- and postmenopausal women, thus we used middle-aged OVX rats in order to model this population of consumers.

2. Materials and methods

2.1. Animals and botanical exposure

A total of 64 middle-aged female Long-Evans rats were obtained from Harlan (Indianapolis, IN). The rats were received in one cohort. All animals 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-h reverse light–dark cycle (lights off at 7:00 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 and the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (NIH, 1986; Van Sluyters and Obernier, 2004).

Rats were 12 month old retired breeders, and were assigned to one of four exposure groups (0,6,12 or 24 mg of ISL). The body weights of the rats ranged from 272 to 373 g (M = 316, SD = 22). Thus, the doses of ISL correspond to approximately 0, 19, 38, or 76 mg/kg body weight. There were 16 rats per group. Rats were pair-housed in standard plastic cages (45 × 24 × 20 cm) with Beta Chip® bedding, and were allowed to acclimate to the vivarium for two weeks before ovariectomy. For the ovariectomy surgery, rats were anesthetized with isoflurane gas. Following removal of the ovaries, muscle and fat layers were individually sutured with silk suture thread (Fisher Scientific). The incision in the skin was then closed with stainless steel wound clips. A postsurgical injection of carprofen (5 mg/kg, s.c.) was administered for pain management. All animals were maintained on an AIN-93G soy-free diet (Harlan-Teklad, Madison, WI) after surgery to avoid exposure to dietary estrogens. Water was available *ad libitum*. Beginning nine days after the OVX surgery, rats were food restricted to reduce them to 85% of their free-fed body weights. Each animal was weighed daily and given a ration of food 30 min after testing. The amount of food given was adjusted daily based on current weight in comparison to free feeding weight. Twelve days after food restriction began, ISL treatment and operant testing began. Rats were tested once per day, 6 days per week during the dark phase of the light cycle in a darkened testing room. One hour prior to testing, rats were given fruit punch flavored pellets (Test Diet, Richmond, IN) containing doses of roughly 0 mg/kg/day, 19 mg/kg/day, 38 mg/kg/day, or 76 mg/kg/day of ISL. Rats were dosed 7 days per week, including on days they were not tested.

2.2. Serum ISL and LIQ concentration analysis

Serum ISL and LIQ levels were measured in a pilot study prior to the current study (Fig. 1). Six Long-Evans rats aged 9–12 months were OVX and fed an AIN soy-free diet for 4–5 weeks. Rats were fed a dosing pellet