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# Behavioral, Physiological, and Molecular Characterization of Long-Term Administration of a Novel Estrogen Receptor Beta Agonist in a Mouse Model of Menopause

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BEHAVIORAL, PHYSIOLOGICAL, AND MOLECULAR CHARACTERIZATION OF

LONG-TERM ADMINISTRATION OF A NOVEL ESTROGEN RECEPTOR BETA

AGONIST IN A MOUSE MODEL OF MENOPAUSE


by

Aaron Fleischer



A Thesis Submitted in

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ABSTRACT

BEHAVIORAL, PHYSIOLOGICAL, AND MOLECULAR CHARACTERIZATION OF
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AGONIST IN A MOUSE MODEL OF MENOPAUSE

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Aaron Fleischer

The University of Wisconsin-Milwaukee, 2021
Under the Supervision of Dr. Karyn Frick

The menopausal loss of circulating hormones, including estrogens, is associated with

negative symptoms, such as hot flashes, anxiety and depression, cognitive decline, and

weight gain. Although estrogenic hormone therapies (HT) prevent many of the negative

symptoms related to the menopausal transition, these same therapies are associated

with increased health risks, such as the development of breast and ovarian cancers,

which is mediated by the activation of the $\alpha$ (ER$\alpha$), but not $\beta$ (ER$\beta$), estrogen receptor

isoform. Furthermore, ER$\beta$ agonism has previously been shown to reduce preclinical

indices of hot flashes, memory decline, anxiety, and depression. As most ER$\beta$ agonists

are only modestly selective for ER$\beta$ over ER$\alpha$, more selective ER$\beta$ agonists are needed

as potential therapeutics for menopausal women. As part of a multi-university

collaboration, we have helped to develop a novel ER$\beta$ agonist, EGX358, which has 750-

fold selectivity for ER$\beta$ over ER$\alpha$ without any off-target receptor activation or inhibition.

Although we have shown that acute treatment with EGX358 enhances memory

consolidation in young ovariectomized (OVX) mice, we have yet to investigate this novel

agonist's effects when administered long-term. In the current study, we orally treated

young, OVX female mice via gavage with vehicle, the highly potent estrogen 17$\beta$-

estradiol ($E_2$), the commercially available ERβ agonist diarylpropionitrile (DPN), or EGX358 for 64 days. Mice were weighed weekly and tested for vasomotor outcomes following peripheral injection with a neurokinin B agonist, anxiety- like behaviors in the open field and elevated plus maze, depression-like behaviors in the tail suspension and forced swim tests, and memory in the object placement and recognition tasks, and measured for baseline tail skin temperature at the end of the study. On the final day of treatment, mice were euthanized 30 minutes following gavage, and tissues from the dorsal hippocampus, medial prefrontal cortex, amygdala, and hypothalamus – regions highly implicated in memory, affect, and vasomotor symptoms – were collected rapidly for subsequent Western blot analyses. Mice treated with $E_2$, DPN, and EGX358 all had reduced drug-induced increases in tail skin temperature relative to vehicle-treated mice and showed greater performance in both memory tasks than chance levels. $E_2$ also reduced anxiety-like behaviors and baseline tail skin temperature relative to vehicle-treated mice. No other treatment effects were noted for anxiety- or depression-like behaviors, or on body weight over time. Surprisingly, there were also no treatment effects on expression or phosphorylation of proteins related to cellular activation, cellular signaling, pre- or postsynaptic strengthening, or sensitivity to glutamatergic signaling in any region examined. Additionally, there were no differences in ERβ levels in the hippocampus or prefrontal cortex, suggesting that loss of sensitivity to treatment was not the reason for a lack of molecular results. Altogether, our results indicate good potential for further development of EGX358 as a new ERβ-selective HT option for menopause-related symptoms, although its molecular mechanisms remain unknown.

**TABLE OF CONTENTS**

## LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| $E_2$ | 17$\beta$-Estradiol |
| DPN | Diarylpropionitrile |
| HT | Hormone Therapy |
| SC | Subcutaneous |
| i.p. | Intraperitoneal |
| ER$\alpha$ | Estrogen Receptor $\alpha$ |
| ER$\beta$ | Estrogen Receptor $\beta$ |
| OVX | Ovariectomy |
| DH | Dorsal Hippocampus |
| mPFC | Medial Prefrontal Cortex |
| OP | Object Placement |
| OR | Object Recognition |
| OF | Open Field |
| EPM | Elevated Plus Maze |
| TST | Tail Suspension Test |
| FST | Forced Swim Test |
| $T_{Skin}$ | Tail Skin Temperature |
| $\Delta T_{Skin}$ | Change in Tail Skin Temperature |
| ERK | Extracellular Signal-Regulated Kinase |
| BDNF | Brain-Derived Neurotrophic Factor |
| GluA | AMPA Receptor Subunit |
| PSD95 | Postsynaptic Density Protein 95 |

ACKNOWLEDGEMENTS

**Introduction**

The menopausal transition, primarily characterized by the loss of circulating hormones such as estrogens, is often associated with many negative symptoms. Among these, hot flashes, anxiety, depression, cognitive decline, and weight gain are cited regularly as potent disruptors to women's quality of life (Bosworth et al., 2001; Bromberger et al., 2013; Epperson et al., 2013; Lovejoy, 1998; Mitchell & Woods, 2001; Woods & Mitchell, 2005). Importantly, these symptoms compound upon one another, further exacerbating the detrimental effects of this transitional life event (Gold et al., 2000; Greendale et al., 2010; Maki et al., 2008; Maki, 2013). Therefore, it is imperative that treatments be developed to prevent and reduce the negative symptoms associated with the menopausal transition. Estrogenic hormone therapies (HTs) are available and have been used successfully in the past to reduce menopausal symptoms, although these treatments are also associated with increases in risks to women's health, such as ovarian and breast cancer development (MacLennan et al., 2004; Maki, 2013; Péqueux et al., 2012; Schmidt et al., 2000). Importantly, many of the detrimental effects of HTs appear to be mediated by the activation of the $\alpha$ (ER$\alpha$), but not $\beta$ (ER$\beta$), estrogen receptor isoform (MacLennan et al., 2004; Péqueux et al., 2012; Schmidt et al., 2000). Therefore, future HTs should specifically target ER$\beta$ in order to provide the beneficial effects of estrogenic treatment while avoiding the deleterious effects of ER$\alpha$ activation. We have recently worked with collaborators to develop and begin testing a promising novel, highly selective ER$\beta$ agonist, EGX358, although the effects of long-term administration of this compound are currently unknown. The objective of this thesis was to test the efficacy of long-term EGX358 administration in an ovariectomized (OVX) mouse model of menopause to

1

reduce preclinical indices of menopause-related symptoms and influence protein expression in ERβ-laden brain regions.

*Review of the Literature: Estrogen-Based Hormone Replacement Therapies*

Premarin, a conjugated equine estrogen, has been among the primary HT options for treating menopausal symptoms decades due to its efficacy in reducing symptom incidence and severity (Grady et al., 1992; Grodstein et al., 1997; Kimura, 1995; Maki, 2013). However, the initial findings from the Women's Health Initiative, the largest study examining the effects of HT in post-menopausal women showed that long-term treatment with equine estrogens and progestin increased the risks of developing coronary heart disease, stroke, pulmonary embolism, and invasive breast cancer (Rossouw et al., 2002). Subsequent work suggests that these side effects are due to activation of ERα, rather than ERβ. For example, single-nucleotide polymorphisms on the ERα gene which facilitate greater binding affinity for the potent estrogen, 17β-estradiol ($E_2$), to ERα, are associated with increased risks for cardiovascular disease, coronary artery disease, and stroke (Alevizaki et al., 2007; Shearman et al., 2003, 2005). Additionally, others have demonstrated that chronic $E_2$ treatment in OVX female wild type and ERβ knockout mice, but not ERα knockout mice, promotes tumor cell growth (Péqueux et al., 2012). Furthermore, Premarin is comprised of more than 50% estrone, which has a higher affinity for binding ERα compared to ERβ (Englund & Johansson, 1978; Perkins et al., 2017). These lines of evidence suggest that the deleterious side effects associated with HT may be mediated by ERα activity.

*Phytoestrogens: An ERβ-Selective Treatment Method*

Phytoestrogens are a class of compounds found in many different plant species, and these compounds interestingly demonstrate selective affinity for ERβ as compared to ERα (Cheng et al., 2007; Nikov et al., 2000). Among these, isoflavones are the most commonly found and used in both preclinical and clinical studies, demonstrating between 8- and 68-fold selectivity for ERβ (Cheng et al., 2007; Nikov et al., 2000). When tested clinically and preclinically, phytoestrogens are indeed capable of reducing menopausal symptoms and even demonstrate efficacy in reducing cancer cell proliferation. For example, 3 months of isoflavone treatment reduced hot flash occurrences by 50% in postmenopausal women, and 12 weeks of treatment with MF101, an ERβ-selective herbal extract, also reduced hot flash incidences (Cheng et al., 2007; Grady et al., 2009). Additionally, ERβ-selective phytoestrogens have been shown to reduce ovarian cancer cell proliferation rates in cell cultures, as well as reduce the growth of tumors in mouse models (Sareddy et al., 2012; Schüler-Toprak et al., 2017). Although these results are promising in terms of developing ERβ-selective treatments, it is difficult to attribute these effects solely to phytoestrogens, given that these compounds are found ubiquitously in commonly consumed foods. Additionally, although phytoestrogens are somewhat selective for ERβ over ERα, greater selectivity will provide more assurance that long-term treatment would not inadvertently target ERα as well. Thus, treatment options with greater selectivity for ERβ are needed.

*EGX358, a Novel, Highly Selective ERβ Agonist*

Synthetic ERβ agonists are readily available for commercial purchase, and these have shown preclinical efficacy for reduction of cancer cell proliferation in cell culture, improvement of memory and anxiety- and depression-like outcomes in rodents (Boulware

et al., 2013; Hanson et al., 2018; Lund et al., 2005; Pereira et al., 2014; Rocha et al., 2005; Walf et al., 2004). It should be noted, however, that these compounds also have relatively low selectivity for ERβ, as the most commonly used agonist, diarylpropionitrile (DPN, Figure 1), is only about 70-fold more selective for ERβ over ERα (Meyers et al., 2001). Therefore, there is a need for ERβ agonists with greater selectivity.

As part of a multi-institutional collaboration, our laboratory has spent the past six years developing a novel, highly selective ERβ agonist, EGX358 (Figure 1). EGX358 closely resembles the structure of the native $E_2$, and has been classified as an "A-C" estrogenic compound, as the "B" cyclohexane and "D" cyclopentane groups have been reduced to simplify the $E_2$ structure into a 4-hydroxymethyl-cyclohexane ring tethered to a phenolic ring (Hanson et al., 2018). EGX358 exhibits 750-fold selectivity for ERβ over ERα, and does not produce off-target effects on androgen, glucocorticoid, mineralocorticoid, peroxisome proliferation-activated, progesterone, thyroid hormone, or vitamin D receptors, thereby making it the most selective synthetic ERβ agonist to date (Hanson et al., 2018). Acute treatment with EGX358 delivered peripherally via oral gavage or intraperitoneal injection, or bilaterally infused directly into the dorsal hippocampus (DH), immediately post-training



**Figure 1. Structure and ERβ Selectivity of EGX358.** Structural comparisons of 17β-estradiol, DPN, and EGX358.

enhances both object placement (OP) and object recognition (OR) memory consolidation in OVX mice (Hanson et al., 2018). However, the effects of long-term treatment of EGX358 are currently unknown. As we continue to develop this compound as a potential therapeutic for menopause-related symptoms, it is imperative to determine the long-term behavioral, physiological, and cellular and molecular effects of this compound in a preclinical model.

*Preclinical Models of Menopausal Symptoms and their Treatment with ERβ Agonists*

The most common method of modeling menopause preclinically is through bilateral ovariectomy of young female rodents. This surgical method allows for the removal of the primary sources of circulating estrogens and progestins. However, as useful as OVX is for eliminating circulating sex hormones, it should be noted that this immediate loss of hormones does not recapitulate the gradual processes of aging and ovarian senescence in menopause, as the majority of women experiencing menopause retain their ovaries (Koebele & Bimonte-Nelson, 2016). Nevertheless, OVX in young mice has been widely adopted in the field as a method to mimic the effects of ovarian hormone loss and resulting disruption to the hypothalamic-pituitary-gonadal axis. Bilateral OVX is capable of increasing anxiety- and depression-like behaviors, while also impairing spatial and object recognition memories relative to sham control surgery in female rodents (Walf & Frye, 2006; Wallace et al., 2006). Although OVX also increases tail skin temperature, a measure of vasodilation used as a proxy of hot flashes, this is a very gradual occurrence that does not model well the rapid experiences of hot flashes, which are driven largely by disrupted neurokinin B signaling in the median preoptic area of the hypothalamus resulting from the loss of estrogenic negative feedback in the arcuate nucleus (Padilla et

al., 2018; Rance et al., 2013; Tan et al., 2016). More recently, a synthetic agonist of tachykinin 3 receptors, which are primarily responsible for the actions of neurokinin B, has been developed. This agonist, senktide, is a potent tachykinin 3 receptor agonist which elicits a pronounced, transient increase in tail skin temperature in intact male and female, as well as OVX female mice (Krajewski-Hall et al., 2018; Krull et al., 2017). Thus, ovariectomy and administration of senktide are effective preclinical methods to produce several symptoms of menopause.

Importantly, the effects of OVX and senktide can be reduced by estrogenic compounds. For example, we and others have demonstrated that acute and long-term administrations of $E_2$, DPN, or phytoestrogens enhance memory function in OVX rodents, particularly in spatial and object recognition tasks (Boulware et al., 2013; Hanson et al., 2018; Jacome et al., 2010; Pereira et al., 2014; L. Zhao et al., 2011). Furthermore, the ability of $E_2$ to reduce anxiety- and depression-like behaviors in OVX rodents appears to be mediated largely by $ER\beta$ activation, as $ER\beta$, but not $ER\alpha$, agonists decrease these behaviors, and genetic knockout of $ER\beta$, but not $ER\alpha$, blocks $E_2$'s anxiolytic and anti-depressive effects (Eid et al., 2020; Lund et al., 2005; Oyola et al., 2012; Walf & Frye, 2006). Although no previous studies have specifically examined the role of $ER\beta$ signaling in senktide-mediated vasodilation, $E_2$ delivered chronically does reduce senktide's effects on vasodilation (Krajewski-Hall et al., 2018). A role for $ER\beta$ is suggested by data from $ER\alpha$ knockout mice, in which $E_2$ reduced gradual increases in baseline tail skin temperature following OVX, and by another study in wildtype mice in which long-term treatment with an $ER\beta$-selective phytoestrogen diet prevented OVX-associated increases

in baseline tail skin temperature (Opas et al., 2006; L. Zhao et al., 2011). These findings suggest a possible role for ERβ signaling in preventing hot flash-like symptoms.

Together, existing evidence suggests that OVX in young mice can reproduce key symptoms of menopause and ERβ activation is a promising method for alleviating these symptoms. Thus, there is a strong basis to suggest that long-term treatment with ERβ agonists, including EGX358, could present a safe and effective method for treating menopausal symptoms.

*Effects of ERβ Activation in the Brain*

The three main estrogen receptors, ERα, ERβ, and G-protein coupled estrogen receptor, are widely distributed throughout the brain and influence neural activity when an estrogenic ligand is present (Alexander et al., 2017; Almey et al., 2014; Dacks et al., 2011; Mitra et al., 2003; Oberlander & Woolley, 2017; Shughrue et al., 1997). In particular, ERβ is found throughout the hippocampus, medial prefrontal cortex (mPFC), amygdala, and hypothalamus, regions highly associated with learning and memory, affective symptoms, and thermoregulation (Almey et al., 2014; Mitra et al., 2003; Shughrue et al., 1997). Thus, these regions are of particular interest in determining the molecular mechanisms by which ERβ agonism might affect menopausal symptoms.

Within the DH, which is implicated in both memory outcomes and affective symptoms, the Frick lab has demonstrated repeatedly that acute bilateral infusion of $E_2$ enhances spatial and object memory consolidation in OVX mice through upregulation of extracellular signal-regulated kinase (ERK) phosphorylation, brain-derived neurotrophic factor (BDNF), and cofilin phosphorylation, among others, which all converge to facilitate synaptic plasticity, as indicated by increased CA1 dendritic spine density in this region

(Buzsáki & Moser, 2013; Fernandez et al., 2008; Gross et al., 2021; J. Kim et al., 2019; Tuscher, Luine, et al., 2016; Zhu et al., 2019). We and others have also shown that acute, bilateral infusion or up to 7 days of peripheral treatment with ER$\beta$ agonists also upregulate plasticity-mediated protein expression and activation in the DH of young and old OVX female mice, respectively; namely phosphorylation of ERK and cofilin, upregulation of the F-actin:G-actin ratio, and increased CA1 spine densities are all results of ER$\beta$ agonism in the DH (Boulware et al., 2013; Y. Zhao et al., 2017). Although few studies have examined the molecular effects of long-term ER$\beta$ activation, at least one study has demonstrated that long-term treatment with ER$\beta$-selective phytoestrogen-rich diets increases BDNF and PSD95 expression in the hippocampus (L. Zhao et al., 2011). Additionally, DPN administered for 29 days resulted in greater GluA2 and GluA3 mRNA in the hippocampi of OVX rats, suggesting a greater sensitivity to glutamatergic signaling in this region due to ER$\beta$ activity (Sárvári et al., 2016). Therefore, the above are all potential targets by which EGX358 might influence memory outcomes.

As with the hippocampus, the mPFC serves a dual role in memory and emotional processing, and this region appears to be highly sensitive to the effects of estrogenic signaling with a potential role of ER$\beta$ activation. We and others have demonstrated this by acutely treating OVX rodents via either peripheral injection or direct bilateral infusion with E$_2$, which increases expression of the immediate early gene c-Fos, a marker of cellular activity, and dendritic spine densities within 2 hours (Tuscher et al., 2019; Zeidan et al., 2011). Additionally, although less is known about the specific role of ER$\beta$ activation in the mPFC, ER$\beta$ are poised to mediate these effects given their localization to mPFC axon terminals, dendritic spines, and glia in rats (Almey et al., 2014). Additionally,

peripheral injection of DPN is sufficient to increase dopamine, serotonin, and their metabolites HVA and 5HIAA, respectively, in the mPFC, and 8 weeks of phytoestrogen-rich diets increase BDNF mRNA expression therein, suggesting involvement of ERβ-specific mechanisms in this region (Jacome et al., 2010; Pan et al., 1999). These ERβ localizations and plasticity-related changes indicate that long-term ERβ agonism may enhance synaptic connectivity in the mPFC in similar ways as in the DH, although much more work is needed to determine the veracity of this speculation.

Much less is known about the molecular mechanisms through which ERβ activity in the amygdala and hypothalamus might influence affective and vasodilatory outcomes, respectively. In the amygdala of OVX rats, $E_2$ infusion into the central or medial nuclei reduces anxiety- and depression-like behaviors, an effect thought to be mediated by ERβ activity (Frye & Walf, 2004; Le Moëne et al., 2019). These behavioral outcomes may result from reduced cellular activity in the amygdala, as $E_2$ treatment in gonadally intact rats in the low-estrogen metestrus phase decreases c-Fos expression (Zeidan et al., 2011). Although this finding suggests that $E_2$ reduces synaptic plasticity in the amygdala, much more needs to be done to investigate a role for ERβ-driven molecular candidates in the reduction of affective behaviors. Similarly, it is known that vasomotor symptoms are associated with increases in c-Fos expression in the median preoptic area and arcuate nucleus of the hypothalamus are associated with increased hot flash-like symptoms and that long-term treatment with $E_2$ in OVX rodents both reduces vasomotor symptoms and prevents elevations in c-Fos in these regions (Dacks et al., 2011; Krull et al., 2017). However, to date, no studies have investigated the roles of ERβ activity in these brain region with respect to hot flash-like indices. Therefore, further investigation is needed to

determine whether and how ERβ agonism might affect this region, especially with regard to vasomotor outcomes.

*Goals of this Thesis*

The primary objective of this thesis was to determine the extent to which long-term oral treatment with EGX358 in OVX mice enhances memory, alleviates senktide-induced vasodilation, reduces anxiety- and depressive-like behaviors, influences body weight, and regulates levels of cell-signaling, synaptic, and growth factor proteins in the DH, mPFC, amygdala, and hypothalamus. Mice were gavaged daily for 64 days with vehicle, $E_2$, DPN, or EGX358, during which time they were tested for senktide-mediated tail skin temperature responses, open field and elevated plus maze indices of anxiety-like behavior, tail suspension and forced swim test indices of depression-like behavior, spatial and object memory as tested in the object placement and object recognition paradigms, and long-term changes in baseline tail skin temperature. On the final day of treatment, mice were euthanized, and tissues from the DH, mPFC, amygdala, and hypothalamus were collected and subsequently analyzed via Western blot for markers of cellular activity (c-Fos), phosphorylation of rapid kinases (p42 ERK and CaMKIIα), neurotrophic factors (BDNF), presynaptic markers of vesicular release of neurotransmitters (SNAP25, synaptophysin), postsynaptic markers of plasticity (PSD95, GluA ratio), and postsynaptic markers of actin remodeling (F-actin, phospho-cofilin), as well as ERβ. Our results indicate that long-term oral EGX358 treatment can reduce senktide-induced vasodilation and enhance both object recognition and object placement memory outcomes to similar extents as $E_2$ and DPN, but that only E2 treatment reduces anxiety-like behaviors and baseline tail skin temperature compared to vehicle treatment. DPN and EGX358 did not

affect anxiety- or depression-like behaviors or baseline tail skin temperature compared to vehicle, and there were no treatment effects on body weight following OVX. Surprisingly, none of the estrogen treatments altered protein levels in any brain regions examined. Thus, although the mechanisms underlying its effects remain elusive, EGX358's beneficial effects on several preclinical indices of menopausal symptoms, without concomitant negative effects on affective behaviors or body weight, make it a promising lead compound for alleviating memory dysfunction and hot flashes in menopausal women.

**Methods and Materials**

*Subjects*

Subjects were eight-week-old female C57BL/6 mice (n=40), obtained from Taconic Biosciences (Germantown, New York). Mice were housed five per cage until 24 h prior to surgery. Following surgery, mice were singly housed for the duration of the experiment. Mice were maintained on a 12-hour light-dark cycle (lights on at 07:00) and were given food and water access *ad libitum*. Diet was standard rodent chow (Teklad Rodent Diet 8604, Envigo, Madison, WI), which contained isoflavone equivalents of daidzein and genistein aglycone ranging from 350 to 650 mg/kg. Handling to acclimate mice to the experimenters began one week after surgery. All treatments and behavioral testing occurred during light hours. All procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

*General Experimental Design*

The experimental timeline for the study is illustrated in Figure 2, with additional

visual representations of the procedures used depicted in Figures 3 and 4. Three days

after arrival in the lab, mice were ovariectomized (Figure 2). Mice were weighed
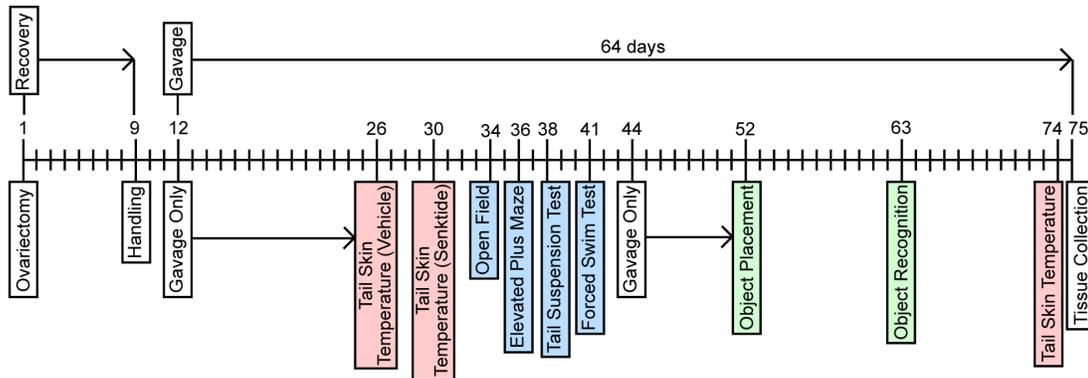


**Figure 2. Experimental timeline.** Young female C57BL/6 mice were bilaterally ovariectomized at approximately 8 weeks of age. Mice recovered for 7-8 days, after which wound clips were removed. The next day, mice began daily oral gavage treatment (10% DMSO, $E_2$, DPN, or EGX358). Daily oral gavage continued for the duration of the experiment, 64 days in total. During the first 2 weeks of treatment, mice were also subcutaneously injected with 0.9% saline every other day. Following the first 2 weeks of treatment, mice began behavioral testing, which was conducted in the following order: $T_{Skin}$ with subcutaneous injection of 0.9% saline (vehicle), $T_{Skin}$ with subcutaneous injection of senktide, open field, elevated plus maze, tail suspension test, forced swim test. After completing the forced swim test, mice were gavaged without behavioral testing for 8 days, after which they began object placement and, subsequently, object recognition training and testing. The day after completion of object testing, mice were thermally imaged for $T_{Skin}$ 1 hour following gavage. On the final day of the experiment, mice were gavaged and euthanized 30 minutes later, and brain tissue was rapidly dissected and stored at -80°C. Mice were gavaged 1 hour prior to each behavioral test. DMSO, dimethyl sulfoxide; $E_2$, 17β-estradiol; DPN, diarylpropionitrile; $T_{Skin}$, tail skin temperature.

immediately prior to surgery and once weekly throughout the remainder of the study to

appropriately dose treatments and to test for effects of treatment on weight gain.

Following recovery from OVX surgery, mice were gavaged daily with their respective

treatments for two weeks in their colony room without being behaviorally tested. During

this two-week period, mice were also given subcutaneous (SC) injections of 0.9% saline

every other day to acclimate them to injection stress prior to vasodilation measurements.

Daily gavage continued throughout the duration of the study, and mice were gavaged in

their colony room 1 h prior to testing on days of behavioral and physiological

measurements, unless otherwise noted. This timepoint was chosen because of the

number and variety of behavioral tasks in the current study. Due to the wide variety of times at which estrogenic compounds elicit behavioral (Bekku & Yoshimura, 2005; Bernardi et al., 1989; Estrada-Camarena et al., 2003; Gresack & Frick, 2006a; Harburger et al., 2007; Li et al., 2014; Lund et al., 2005; Oyola et al., 2012) and neural effects (Eid et al., 2020; Galea, 2008; Inagaki et al., 2010, 2012; Jacome et al., 2010; Phan et al., 2012) when administered peripherally to ovariectomized rats and mice, and given that the time frame in which systemically-administered EGX358 activates ER$\beta$ in the brain is currently unknown, one hour was chosen to allow sufficient time for the compound to cross the blood-brain barrier and bind ER$\beta$ in target brain regions.

Mice were first tested for vasodilation responses to vehicle, and then senktide, administration. Next, anxiety-like behaviors were measured in the open field (OF) and elevated plus maze (EPM). Then, depression-like behaviors were measured in the tail suspension test (TST) and forced swim test (FST). Following completion of FST, mice did not undergo behavioral testing for one week to mitigate potential effects of accumulated stress on memory tests. Then, mice were trained and tested in OP and OR to assess memory. Finally, mice were thermally imaged one day following memory testing to determine long-term effects of treatment on baseline thermal regulation. Following completion of all measurements, mice were gavaged and euthanized 30 min later, and tissue from ER$\beta$-laden brain regions implicated in menopause-related symptoms was rapidly dissected and collected on wet ice and frozen at -80°C for follow-up biochemical studies at a later date.

All behavioral tasks were conducted in lighting <100 lux and ambient room temperature of 23 $\pm$ 2°C. To reduce the chances of mice associating a given behavioral

testing room with stressful situations, tests were conducted in three separate rooms: vehicle and senktide vasodilation measures, TST, and FST were conducted in one; OF, OP, and OR were conducted in a second; and EPM was conducted in the third. The final gavage and euthanasia were conducted in a fourth room. Thermal images and all gavage treatments prior to euthanasia were conducted in the colony room. Additionally, during all behavioral test sessions, a researcher blind to treatment initiated recordings in rooms adjacent to those in which the behavior was being conducted, and researchers remained outside of the testing rooms during testing. All behavioral and physiological measurements were quantified and recorded by researchers blind to treatment following completion of a given task, unless otherwise noted.

*Ovariectomy Surgery*

Mice were singly housed 24 h prior to surgery. On the day of surgery, mice were bilaterally ovariectomized using a procedure that our laboratory has repeatedly employed to eliminate circulating estrogens (Frick & Berger-Sweeney, 2001; Gresack & Frick, 2006b; Hanson et al., 2018; Koss et al., 2018; Tuscher, Luine, et al., 2016), as previously verified by vaginal lavage (Frick & Berger-Sweeney, 2001). Briefly, on the day of surgery, mice were anesthetized with isoflurane (5% for induction, 2% for maintenance). Immediately after anesthesia induction, mice were given a SC injection of Rimadyl (1:100, 10 mL/kg) for analgesia. Mice were then shaved bilaterally on their flanks and incised, after which their ovaries, oviducts, and tips of the uterine horns were ligated with monofilament and then removed, as confirmed by visual observation. Muscle wall incisions were sutured with chromic gut, and skin incisions were closed with wound clips. Mice recovered for seven days, the first two days of which they were given SC Rimadyl

injections for post-operative analgesia. Following this recovery period, wound clips were removed, and mice were replaced in their colony room.

*Drugs and Administration*

Following recovery from surgery and wound clip removal, mice were handled briefly (30 s/day) for three days to habituate to experimenter handling (Figure 2). On the fourth day, mice began daily gavage treatment (n=10/group) in the colony room using bulb-tipped gastric gavage needles (24 GA, 25 mm) (Hanson et al., 2018). Mice were gavaged daily for two weeks prior to behavioral testing and throughout the rest of the study. On days of physiological
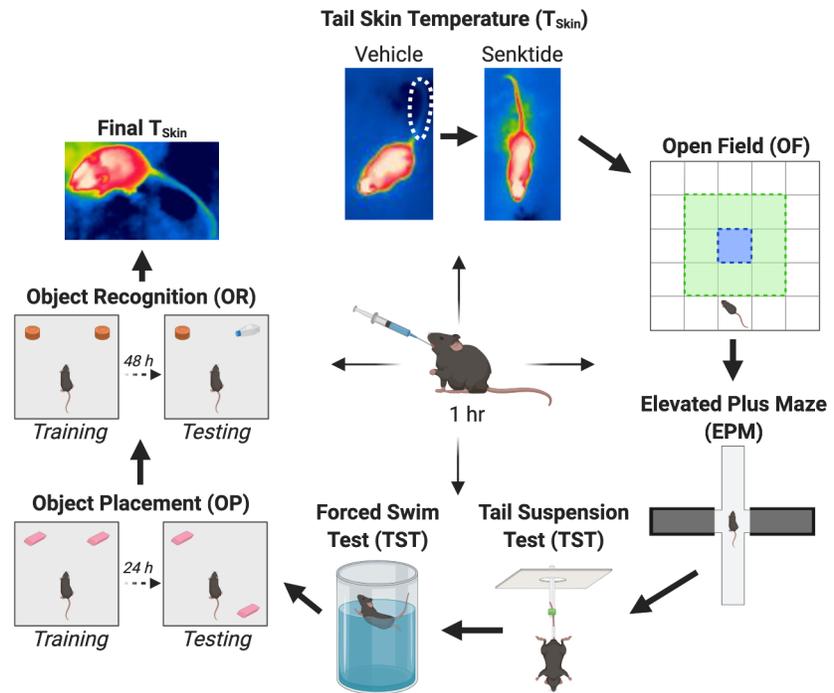


**Figure 3. Visual representations of behavioral tasks.** Young, ovariectomized mice were gavaged with 10% DMSO, $E_2$, DPN, or EGX358 1 hour prior to each behavioral or physiological measurement. Diagrams represent each behavioral apparatus. Mice were thermally imaged for tail skin temperature following subcutaneous injection of 0.9% saline and, subsequently, senktide for 30 minutes. Next, mice were measured for anxiety-like and locomotor behaviors in the open field, namely time spent in and entries into each zone, as well as distance traveled during the 10-minute test. Mice were then measured for anxiety-like behaviors in the elevated plus maze for 10 minutes, quantified as entries into and time spent in each set of arms and the center space. Mice were measured for time spent immobile and latency to first bout of immobility as proxies of depression-like behaviors in the tail suspension test for 6 minutes and, subsequently, the forced swim test for 4 minutes. Mice were tested for memory consolidation in the object placement and object recognition tasks by measuring time spent with the moved or novel objects, respectively, during the testing phase of each task. Finally, mice were thermally imaged for tail skin temperature 1 hour after gavage on the final day of the experiment. DMSO, dimethyl sulfoxide; $E_2$, 17$\beta$-estradiol; DPN, diarylpropionitrile. Created with BioRender.com.

measurements and behavioral training and testing, mice were gavaged 1 h prior to measurement (Figure 3). Mice received β-cyclodextrin-encapsulated $E_2$ (Sigma-Aldrich, 0.2 mg/kg), DPN (Tocris Biosciences, 0.05 mg/kg), or EGX358 (0.5 mg/kg), at doses previously shown to enhance hippocampus-dependent memory consolidation when administered systemically (Gresack & Frick, 2006; Hanson et al., 2018). Given the hydrophobicity of EGX358 (Hanson et al., 2018), we have previously dissolved this compound in 10% DMSO when acutely treating mice peripherally via gavage or intraperitoneal injection (Hanson et al., 2018). Thus, each treatment in the current study was dissolved in 10% DMSO in 0.9% saline, and all treatments were administered at 10 mL/kg, as described previously (Hanson et al., 2018). Vehicle-treated mice received 10% DMSO in 0.9% saline daily. To acclimate the mice to injection stress prior to vasodilation measurements, mice were given a SC injection of 0.9% saline (5 mL/kg) every other day during the two weeks prior to behavioral testing. During vasodilation experiments, mice were given a single SC injection of either vehicle (0.9% saline, 5 mL/kg) or senktide (Tocris Biosciences, 0.5 mg/kg, 5 mL/kg) dissolved in 0.9% saline.

*Tail Vasodilation Response*

To measure the effects of long-term treatment on vasomotor symptoms, tail skin temperature ($T_{Skin}$), a proxy for vasodilation (Figure 3), was measured three times, as described previously (Krajewski-Hall et al., 2018; Krull et al., 2017; Padilla et al., 2018). On days of vehicle or senktide challenge, each mouse was transported to a testing room immediately following gavage and given 1 h to acclimate to the new environment prior to testing. In its home cage, each mouse was placed in a secondary container (10 in x 18 in x10 in) beneath a thermal camera (E8, FLIR, Wilsonville, OR, USA). After acclimation,

the cage top was removed and a researcher in an adjacent room initiated continual thermal imaging (FLIR Tools+). Baseline $T_{Skin}$ was recorded for 10 min to ensure there were no effects of stress or activity due to removal of the cage top. Then, a researcher removed the mouse from its home cage and injected the mouse SC with either vehicle or senktide. The mouse was then returned to its home cage, and $T_{Skin}$ was recorded for an additional 20 min. After testing, the cage top was placed back onto the cage, and the mouse was removed from the testing room. On the final experimental day of the study, mice were imaged one time in their colony room 1 h following gavage to determine the effects of long-term treatment with $E_2$, DPN, or EGX358 treatment on $T_{Skin}$ following ovariectomy.

Following completion of behavioral testing, $T_{Skin}$ from these experiments was quantified using FLIR Tools+ software. $T_{Skin}$ was measured by averaging the temperature of the tail in a 1-cm line beginning 2 cm from the tail base, as described by others (Krull et al., 2017). During the vehicle and senktide challenges, the researcher quantified $T_{Skin}$ immediately following the removal of the cage top, as well as at 5 and 7.5 min following cage top removal to ensure that temperature changes were not due simply to stress or increased activity associated with the removal of the cage top. Beginning immediately following SC injection, $T_{Skin}$ was measured every minute for 20 min to assess the acute effects of vehicle or senktide administration. When a tail was obscured at the exact minute of the recording, $T_{Skin}$ was measured at the next full second when the tail was exposed. Mice were analyzed for change in $T_{Skin}$ ($\Delta T_{Skin}$) due to injection, which was calculated via the following equation: ($T_{Skin, Raw} - T_{Skin, Baseline}$), where $T_{Skin, Raw}$ was the $T_{Skin}$ quantification at a given time point, and $T_{Skin, Baseline}$ was the $T_{Skin}$ at 7.5 min following cage top removal,

such that changes in temperature were normalized to the temperature measured most immediately prior to injection (Krajewski-Hall et al., 2018; Krull et al., 2017). Images captured on the last day of the experiment were quantified for $T_{Skin}$. Previously, Krajewski-Hall et al. (2018) utilized a threshold of 30°C, above which mice were removed from analyses of senktide-induced $T_{Skin}$ measurements. Based upon our own preliminary studies, we determined that $T_{Skin}$ exceeding 27°C prior to peripheral injection is indicative of measurement-induced stress and/or increased activity (data not shown), thereby obfuscating effects of senktide on tail vasodilation as measured by thermal imaging. Therefore, based on this previous literature and our own preliminary studies, mice were removed from vehicle (n=1, DPN) or senktide (n=1, DPN) analyses if their $T_{Skin, Baseline}$ was above 27°C (Krajewski-Hall et al., 2018). Additionally, mice were removed from senktide challenge analyses if they did not exhibit tail rattles or cold-seeking behaviors, such as pushing bedding aside to expose the cool bottom of the cage (n=1, Vehicle), both of which are stereotypical responses to senktide treatment (Krajewski-Hall et al., 2018; Krull et al., 2017).

*Open Field Test*

The open field (OF) test assessed locomotor and anxiety-like behaviors (Li et al., 2014; Lund et al., 2005; Oyola et al., 2012; Rocha et al., 2005). The OF arena consisted of an empty white testing box (60 cm x 60 cm x 47 cm) that was divided into a 5 x 5 grid of squares (12 cm x 12 cm). This grid included an outer zone (16 squares total), a middle zone (8 squares total), and a center zone (1 square total) (Figure 3). Mice were transported to a holding room immediately following gavage and remained in the room for 1 h prior to testing to acclimate to a new environment. Mice were placed in the lower

center of the OF arena, facing the bottom wall, and given 10 min to explore. ANY-maze software (Stoelting) automatically tracked the center of each mouse's body and quantified distance travelled in, time spent in, and number of entries into each zone. The number of fecal boli in each zone, as well as the number of bouts and time spent both grooming/barbering and rearing, were manually quantified. After testing, each mouse was immediately returned to its home cage and removed from the testing room, and the OF arena was cleaned with 70% ethanol between each test.

*Elevated Plus Maze*

The elevated plus maze (EPM) was also used to measure anxiety-like behavior (Frick et al., 2000; Lund et al., 2005; Oyola et al., 2012). The open arms of the EPM apparatus (30 cm x 5 cm) consisted of an opaque white Plexiglas floor with a clear lip (0.5 cm) attached to the sides of each arm to prevent mice from falling off the apparatus (Figure 3). The closed arms (30 cm x 5 cm x 15 cm) consisted of opaque black Plexiglas walls and a gray floor. Mice were transported to a holding room, gavaged, and given 1 h to acclimate to the new environment before moving to a testing room. Then, each mouse was placed in the EPM. Mice were placed in the center of the maze, facing the upper open arm, and behavior was recorded via ANY-maze for 10 min. Following completion of EPM testing, the number of fecal boli, number of entries into, and time spent in each set of arms and the center zone were quantified. An entry into either set of arms was determined as a mouse having all four paws within an arm. Otherwise, mice were considered to be in the center zone. Time spent peeking into open arms was also quantified, defined as any time the mouse either oriented its head and up to three paws into an open arm or was looking over the side of the apparatus. Mice were immediately

placed back into their home cages and removed from the room after testing, and the EPM was cleaned with soap and water between each test. One mouse (EGX358 group) was removed from analyses because it fell off of the apparatus during the test.

*Tail Suspension Test*

The tail suspension test (TST) assessed depression-like behavior (Bernardi et al., 1989; Can et al., 2011). The TST apparatus was made of opaque white Plexiglas and consisted of three chambers (10 in x 10 in x 18 in) (Figure 3). A tube was threaded through a hole in the ceiling of each chamber. C57BL/6 mice are known to climb their tails when suspended in this manner, making it difficult to quantify mobility and immobility behaviors (Can et al., 2011). Therefore, a small piece of tubing was placed around the base of each mouse's tail to effectively eliminate tail climbing (Can et al., 2011). Each mouse was suspended in the chamber by its tail, which was taped to the tube 1 cm from the tail tip and with the mouse's ventral side facing the camera. The mouse's behavior was recorded via ANY-maze for 6 min. Following testing, tubing was removed from each mouse's tail, and mice were returned to their home cages and removed from the room. The TST apparatus was cleaned with soap and water between each test. After testing, the number of fecal boli, time spent immobile, and latency to first bout of immobility were measured. Immobility was defined as the mouse remaining motionless or making only those movements needed to reorient its body, such as small front paw movements, slow adjustments, or sniffing. Mobility was defined as additional movements, such as full-body movement, rear paw movement, forceful front paw movement, and the mouse climbing the fur on its abdomen. To ensure accurate scoring, all tests were scored by a single researcher blind to treatment, and each test was scored until two independent immobility

scores were within 3 sec of each other. The average of these two scores was used as the immobility score for each mouse.

*Forced Swim Test*

The forced swim test (FST) was used to measure depression-like behavior (Bekku & Yoshimura, 2005; Can et al., 2011; Estrada-Camarena et al., 2003; Li et al., 2014; Okada et al., 1997; Rocha et al., 2005). The FST apparatus consisted of a glass cylinder (14 in x 6 in diameter) filled with water (20 cm depth) to prevent mice from supporting themselves by touching the cylinder bottom with their paws (Figure 3). Water temperature was measured between each test and maintained at 23±2 °C, and water was changed after three mice completed testing. During the FST, each mouse was gently placed into the water, such that its head was not submerged, and swimming behavior was recorded for 6 min in ANY-maze. After testing, the mouse was removed from the cylinder, dried with paper towels, and returned to its home cage. The home cage was then removed from the testing room and placed on a heating pad for 20 min. The number of fecal boli was recorded and cleaned from the glass cylinder after each mouse completed the FST. The first 2 min of each recording were used to acclimate each mouse to the water. The last 4 min were scored for time spent immobile and latency to first bout of immobility by a single researcher who was blind to treatment groups. Immobility was defined as the lack of movement (floating) or only those movements necessary to rectify the mouse's upright position. Mobility was defined as additional movements, such as swimming and attempting to climb the cylinder wall. As with the TST, each test was scored until two independent immobility scores were within 3 sec of each other. The average of the two scores was used as the immobility score for each mouse.

*Object Placement and Object Recognition*

The object placement (OP) and object recognition (OR) tests (Figure 3) assessed spatial and object recognition memory, respectively (Boulware et al., 2013; Fernandez et al., 2008; Fortress, et al., 2013a; Fortress, et al., 2013b; Hanson et al., 2018; Kim et al., 2016; Tuscher et al., 2016). Briefly, one week after the FST, a Lego Duplo block was placed in the home cage of each mouse for three days to expose it to an object. Then, each mouse was habituated to an empty OF box (60 cm x 60 cm x 47 cm) for 5 min/day for two days. The day after the second habituation, the mouse was placed in the OF box for 2 min before training with objects. After the 2 min accustomization phase, the mouse was returned to its home cage while two identical objects were positioned 5 cm from the upper left and right corners of the OF arena. The mouse was then returned to the OF box and given 20 min to accumulate 30 s of exploration time with the objects, defined as any time when the mouse was adjacent to a given object with its nose and/or front paws directed at and/or touching the object. Exploration was scored live in ANY-maze by a researcher blind to treatment. Mice underwent OP training and testing prior to OR training and testing.

OP and OR were tested 24 h and 48 h post-training, respectively. At these time points, OVX mice treated acutely with $E_2$, DPN, or EGX358, but not vehicle, immediately post-training spend significantly more time than chance (15 s) with the moved or novel objects, respectively (Boulware et al., 2013; Hanson et al., 2018; Tuscher et al., 2019; Kim et al., 2016). During both OP and OR testing, as in training, mice were given 20 min to accumulate 30 s of exploration between the objects, as scored live in ANY-maze. Mice that remember the location and identity of the training objects should spend significantly

more time than chance with moved and novel objects in OP and OR, respectively. Following testing, each mouse was removed from the OF arena and returned to its home cage. The OF arena was cleaned with 70% ethanol between mice after each portion of both OP and OR. Mice were removed from OP (n=1, DPN; n=2, EGX358) or OR (n=1, Vehicle; n=1, DPN) analyses if they did not reach > 27 s of exploration time with objects during the training phase and 30 s during the testing phase.

*Western Blotting*

On day 64 of treatment, mice were euthanized via cervical dislocation and decapitation 30 min following gavage (Figure 4). Tissues from the DH, mPFC, amygdala, and hypothalamus were rapidly collected bilaterally from brain block slices on wet ice and frozen and stored at -80°C. Tissues from each brain region were homogenized and processed via Western blot as described previously (J. Kim et al., 2016; Koss et al., 2018). Briefly, samples were resuspended 1:25 w/v in lysis buffer solution and immediately homogenized via a probe sonicator (Branson Sonifier 250). Next, homogenates were electrophoresed on Tris-Glycine eXtended (TGX) stain-free precast gels (Bio-Rad) and transferred rapidly to polyvinylidene fluoride (PVDF) membranes using a TransBlot Turbo system (Bio-Rad). Membranes were blocked with either 5% or 10% milk suspended in Tris-buffered saline with Tween 20 (T-TBS) and then incubated with primary antibodies (anti-phospho-ERK1/2, anti-phospho-cofilin, anti-phospho-CaMKII, anti-GluA1, anti-F-actin, anti-PSD95, anti-SNAP25, anti-c-Fos, anti-synaptophysin 1:1000, Cell Signaling Technology; anti-ERβ 1.0 μg/mL, Fisher Scientific; anti-BDNF 1:1000, Abcam) overnight

at 4°C. The following day, blots were incubated at room temperature for 1 h in rabbit or
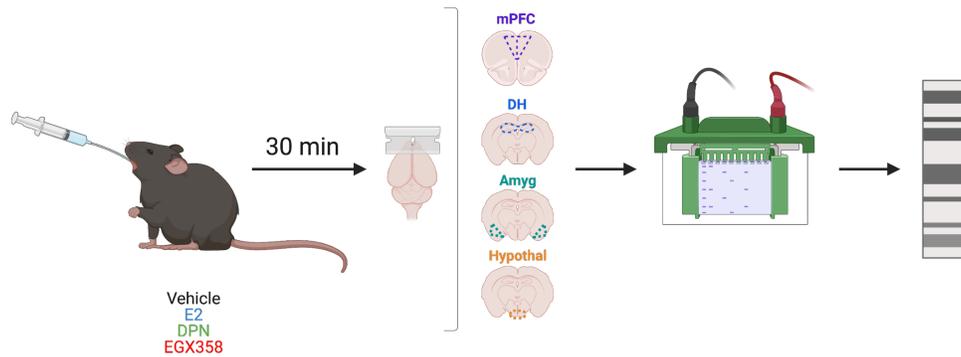


**Figure 4. Visual representation of tissue collection and Western blot analysis.** On day 64 of treatment, mice received either DMSO (vehicle), $E_2$, DPN, or EGX358 and were euthanized 30 min later. Tissues were rapidly collected via brain block slices from the mPFC, DH, amygdala, and hypothalamus and stored at -80°C until Western blot analysis. Tissue homogenates were analyzed for ERK, cofilin, CaMKII, and BDNF signaling, as well as c-Fos, PSD95, F-actin, synaptophysin, SNAP25, and ERβ levels, and/or the ratio of GluA1:GluA2/3/4, depending upon the brain region being examined. DMSO, dimethyl sulfoxide; $E_2$, 17β-estradiol; DPN, diarylpropionitrile. mPFC, medial prefrontal cortex; DH, dorsal hippocampus; Amyg, amygdala; Hypothal, hypothalamus; ERK, extracellular signal-regulated kinase; CaMKII, calcium/calmodulin-dependent kinase II; BDNF, brain-derived neurotrophic factor; PSD95, postsynaptic density protein 95; F-actin, filamentous actin; ERβ, estrogen receptor β; GluA, AMPA receptor subunit. Created with BioRender.com.

mouse HRP-conjugated secondary antibody (1:5000, Cell Signaling Technology), and

developed with West Dura chemiluminescent substrate (Pierce). A ChemiDoc MP gel

imager (Bio-Rad) was used to detect signal correlating with protein expression.

Membranes were then stripped twice via 0.2M NaOH washes, re-blocked with 5% or 10%

milk, and subsequently incubated with primary antibodies (anti-total-ERK1/2, anti-total-

cofilin, anti-total-CaMKII, anti-GluA2/3/4, anti-β-actin 1:1000, Cell Signaling

Technologies) at 4°C and again imaged the next day. Densitometry was performed for

each protein using Image Lab software (Bio-Rad). Phosphorylated protein expressions

were normalized to their total protein expression, GluA1 expression was normalized to

GluA2/3/4 expression, and all other proteins were normalized to β-actin expression. Data

are presented as the immunoreactivity relative to vehicle controls for each protein target,

and treatment effects were measured within single gels (n=3-5/group/gel). The DH and

mPFC were analyzed for phosphorylation of p42 and p44 ERK, cofilin, and CaMKIIα, the ratio of GluA1 to GluA2/3/4, and the total expressions of F-actin, PSD95, SNAP25, c-Fos, synaptophysin, BDNF, and ERβ; the amygdala was analyzed for phosphorylation of p42 and p44 ERK and the total expressions of F-actin, PSD95, c-Fos, synaptophysin, and BDNF; and the hypothalamus was analyzed for total expression of c-Fos.

*Statistical Analyses*

Prior to statistical analyses of treatment effects, outliers, assessed as any data points > 2 standard deviations from the group mean, were removed from each data set. Task-specific exclusions are described above. Finally, one $E_2$-treated and two DPN-treated mice were removed prior to the conclusion of the study and euthanized due to declining health, as indicated by aberrant amounts of barbering or > 5% weight loss between weeks. Therefore, sample sizes are not equal across all test parameters in each measurement, although sample sizes are noted in-text.

All statistical tests were conducted using GraphPad Prism 8 (La Jolla, CA). All anxiety- and depression-like, final $T_{Skin}$, and Western blot measurements were analyzed by one-way analysis of variance (ANOVA), followed by Tukey *post hoc* tests on main effects of group. Body weight measurements and $\Delta T_{Skin}$ measurements during vehicle and senktide challenge were analyzed by mixed-effects two-way ANOVA (treatment x time since OVX, and treatment x time since SC injection, respectively) due to removal of outliers at individual time points, followed by Tukey *post hoc* tests. For memory in the OP and OR tests, two analyses were performed. To assess memory within each group, one-sample *t* tests were used to compare time spent with the moved or novel object, respectively, to chance (15 s). One-way ANOVAs were conducted to compare treatment

25

groups to one another, followed by Tukey's *post hoc* tests on main effects of treatment. Significance was defined at $p < 0.05$. Effect sizes were calculated as Cohen's d for significant *t* tests and eta squared ($\eta^2$) for significant ANOVAs.

**Results**

*Long-Term Oral Administration of a Highly Selective ERβ Agonist, EGX358, did not Adversely Affect Overall Health or Body Weight*

We first sought to ensure that long-term administration of EGX358 does not cause adverse health effects, as measured by disheveled physical appearance or premature death. Young female mice were OVXed (n=40). Following recovery from surgery (Figure 2), mice were gavaged with vehicle (n=10), $E_2$ (n=10), DPN (n=10), or EGX358 (n=10) for 9 weeks. Throughout the duration of this study, three mice were removed due to excessive barbering, weight loss, or poor apparent health. Two DPN-treated mice were removed and euthanized due to weight loss and poor physical appearance, and one $E_2$-treated mouse was removed and euthanized due to excessive barbering, but no vehicle- or EGX358-treated mice were removed from this study. The lack of poor outcomes in the EGX358 group suggests that long-term oral administration of EGX358 has no negative impact on overall health, as observed by physical appearance and measured by weight status.

Mice were also weighed weekly to adjust treatment volume and measure effects of long-term treatment with EGX358 on weight gain following OVX, a commonly documented phenomenon post-OVX that is reduced by $E_2$ when compared to vehicle treatment (Couse & Korach, 1999; Sibonga et al., 1998). Mixed-effects two-way ANOVA

(treatment x time since OVX) analysis revealed a significant effect of time ($F_{(4.102, 138.7)}$ = 103.6, $p < 0.0001$, $\eta^2$ = 0.8815), but not of treatment ($F_{(3, 36)}$ = 0.5673, $p$ = 0.6401). Although the time x treatment interaction was significant ($F_{(30, 338)}$ = 1.644, $p = 0.0202$, $\eta^2 = 0.1023$), Tukey's *post hoc* tests indicated no significant between-group differences at any time point (Figure 5). This interaction appears to be driven by the DPN-treated group, which

**Masses**
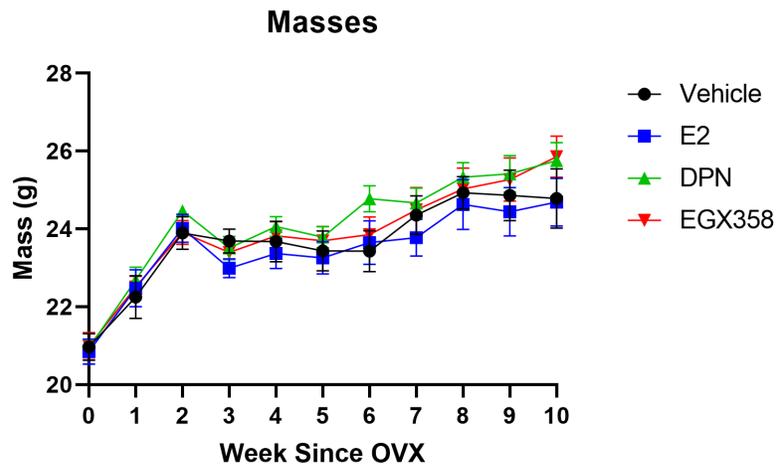


**Figure 5. Long-term treatment with EGX358 did not alter weight gain following ovariectomy.** Young female C57BL/6 mice were bilaterally ovariectomized and given 1 week to recover. Following recovery, mice began daily oral gavage treatment with vehicle (10% DMSO; n=10), $E_2$ (n=10), DPN (n=10), or EGX358 (n=10). Daily treatment continued throughout the duration of the experiment, 64 days in total. Mice were weighed immediately prior to ovariectomy surgery and at the beginning of every week thereafter to both adjust treatment volume, as well as examine the effects of long-term EGX358 treatment on body weight following ovariectomy. There was a significant increase in body weight over the course of the experiment ($p < 0.0001$) that did not significantly differ among the groups at any time point. DMSO, dimethyl sulfoxide; $E_2$, 17β-estradiol; DPN, diarylpropionitrile. Error bars represent mean ± SEM.

weighed somewhat more on average than the other groups beginning 6 weeks post-OVX, although the lack of significant *post hoc* analyses shows this to be a rather weak trend. Collectively, these results suggest that long-term oral EGX358 administration did not adversely affect overall health, nor did it affect weight gain relative to vehicle treatment.

*EGX358, DPN, and $E_2$ Similarly Alleviated an Acute Drug-Induced Increase in Tail Skin Temperature*

Although our group has shown that acute post-training administration of EGX358 promotes memory consolidation in OVX mice (Hanson et al., 2018), we have yet to study

its effects on other preclinical indices of menopausal symptoms. Here, we aimed to determine whether long-term oral treatment with EGX358 at the lowest memory-enhancing dose observed in our previous study could reduce hot flash-, anxiety-, and depression-like symptoms and enhance memory formation. Previous studies have demonstrated that long-term treatment with $E_2$ or ERβ-selective phytoestrogen diets can prevent increases in $T_{Skin}$ in rats and mice following OVX (Opas et al., 2004; L. Zhao et al., 2011), and that this effect is seen in ERα knockout mice (Opas et al., 2006). However, this model does not recapitulate the sudden and transient nature of hot flashes, which are driven by disrupted neurokinin B signaling in the median preoptic area due to the loss of circulating estrogens (Padilla et al., 2018; Rance et al., 2013; Tan et al., 2016). A more recently developed method for vasomotor symptom induction is the administration of a tachykinin 3 receptor agonist, senktide, which elicits a transient increase in $T_{Skin}$ that is prevented by chronic $E_2$ administration (Krajewski-Hall et al., 2018; Krull et al., 2017). Here, we used this method to model hot flash-like symptoms and their treatments.

Sample sizes at the start of the study were n=10/group. As described above and where appropriate in the following sections, some mice were excluded from data analyses due to various issues. As such, sample sizes below reflect the number of mice in each group included in the data analyses. Eleven days after ovariectomy, mice began oral gavage treatment. After two weeks of treatment, mice gavaged with vehicle, $E_2$, DPN or EGX358 were then injected SC with vehicle solution 1 h later to determine possible effects of injection procedures on $T_{Skin}$ (Figures 2, 3). Sample sizes were as follows: vehicle (n=10), $E_2$ (n=10), DPN (n=7), and EGX358 (n=10). As others have demonstrated that ambient room temperature affects $T_{Skin}$ in OVX mice (Krajewski-Hall et al., 2018), the

testing room temperature was measured at the beginning of each test. During testing,

**A  Change in Tail Temperature (VEH)**



room temperature ranged between 22.0 and 22.5°C. In the vehicle challenge, a modest and transient increase in $T_{Skin}$ relative to baseline ($\Delta T_{Skin}$) was observed immediately after SC injection that did not significantly differ between groups (Figure 6A). Mixed-effects two-way ANOVA (treatment x time since SC injection) revealed a main effect of time ($F_{(4.731, 154.3)}$ = 9.169, $p < 0.001$, $\eta^2$ = 0.2959), but no main effect of treatment ($F_{(3, 34)}$ = 1.070, $p = 0.3746$). Although the time x treatment interaction was significant ($F_{(69, 750)}$ = 1.393, $p = 0.0227$, $\eta^2$ = 0.6557), Tukey's *post hoc* tests indicated no significant between-group differences at any time point, despite modestly higher $\Delta T_{Skin}$ in EGX358-treated mice between 5-15 min following SC injection. These results suggest that the injection process itself caused a modest, but

**B  Change in Tail Temperature (SENK)**



**C  Tail Temperature - Final Temperature**
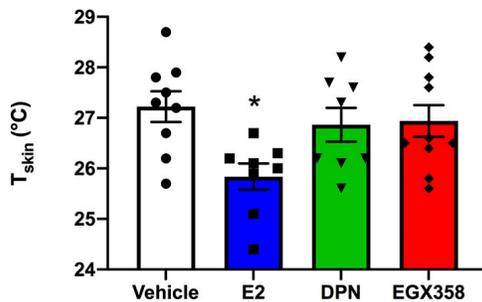


Figure 6. Legend on next page.

transient, increase in $T_{Skin}$ that was not significantly influenced by $E_2$ or ERβ agonist treatment.

**Figure 6. Long-term treatment with EGX358 reduced senktide-mediated increases in tail skin temperature**. Following 2 weeks of daily gavage with vehicle (n=10), $E_2$ (n=10), DPN (n=8), or EGX358 (n=10), mice were evaluated in a senktide-mediated model of vasodilation. Mice were tested twice, once after being injected subcutaneously with 0.9% saline and once after senktide injection, to ensure that senktide-mediated $\Delta T_{Skin}$ could not be attributed to the injection process. A) Baseline $T_{Skin}$ was thermally imaged for 10 min, after which mice were injected subcutaneously with 0.9% saline and then were imaged for another 20 min. Compared to the baseline $T_{Skin}$ measured 2.5 min prior to injection, subcutaneous injection caused a modest increase in $T_{Skin}$ that returned to baseline within 20 min in all treatment groups ($p < 0.0001$). An interaction between treatment and time since injection ($p = 0.0227$) was observed, such that EGX358-treated mice tended be modestly warmer than other mice at 5-15 min post-injection, although *post hoc* analyses revealed no significant differences between treatment groups at any time point. B) In all treatment groups, subcutaneous injection of senktide caused a transient, significant increase in $T_{Skin}$ compared to baseline ($p < 0.0001$). However, an effect of treatment ($p = 0.0041$) and an interaction between treatment and time since injection ($p < 0.0001$) were observed. $E_2$, DPN, and EGX358 reduced $\Delta T_{Skin}$ due to injection compared to vehicle, such that $E_2$-treated mice had significantly lower $\Delta T_{Skin}$ compared to vehicle-treated mice 3-5, 8-12, and 19 min post-injection (blue lines, *$p < 0.05$); DPN-treated mice had significantly lower $\Delta T_{Skin}$ compared to vehicle 8-11, 13, and 19 min post-injection (green lines, *$p < 0.05$); and EGX358-treated mice had significantly lower $\Delta T_{Skin}$ due to injection compared to vehicle 5-9, 16, and 19 min post-injection (red lines, *$p < 0.05$). C) $T_{Skin}$ was measured on the final day of the experiment to determine the effects of long-term treatment on baseline $T_{Skin}$. $E_2$-treated mice had significantly lower $T_{Skin}$ compared to vehicle-treated mice after 63 days of treatment ($p = 0.0221$). $E_2$, 17β-estradiol; DPN, diarylpropionitrile; $T_{Skin}$, tail skin temperature; $\Delta T_{Skin}$, change in tail skin temperature relative to baseline. Error bars represent mean ± SEM. *$p < 0.05$, compared to vehicle.

Four days later, mice were gavaged and injected SC with senktide 1 h later (Figures 2, 3). Again, room temperature was measured at the beginning of each senktide test, ranging between 21.4 and 22.1°C. Mixed-effects two-way ANOVA (treatment x time since injection) revealed main effects of time ($F_{(6.052, 188.4)} = 147.7$, $p < 0.0001$, $\eta^2 = 0.8560$) and treatment ($F_{(3, 32)} = 5.381$, $p = 0.0041$, $\eta^2 = 0.0155$), and a time x treatment interaction ($F_{(69, 716)} = 1.895$, $p < 0.0001$, $\eta^2 = 0.1252$; Figure 6B). *Post hoc* tests revealed that $E_2$-treated mice had significantly reduced $\Delta T_{Skin}$ (change in $T_{skin}$ relative to pre-injection baseline $T_{Skin}$) compared to vehicle-treated mice 3-5, 8-12, and 19 min post-injection ($p < 0.05$). DPN significantly reduced $\Delta T_{Skin}$ compared to vehicle 8-11, 13, and 19 min post-injection ($p < 0.05$). Finally, EGX358 significantly reduced $\Delta T_{Skin}$ compared to vehicle 5-9, 16, and 19 min post-injection ($p < 0.05$). No significant differences among the $E_2$, DPN, or EGX358 groups were revealed at any time point in this test. These results demonstrate

similarly beneficial efficacy of $E_2$, DPN, and EGX358 in reducing vasodilation induced by activation of neurokinin B receptors.

Finally, we examined whether long-term treatment could prevent the gradual increase in $T_{Skin}$ associated with long-term OVX (Opas et al., 2004; Rance et al., 2013; L. Zhao et al., 2011). The day following completion of OR testing (day 63 of treatment), mice were gavaged and thermally imaged in their home cages 1 h later (Figures 2, 3). Sample sizes were as follows: vehicle (n=9), $E_2$ (n=8), DPN (n=8), and EGX358 (n=10). Throughout treatment and imaging, the temperature of the colony room in which observations were made was measured and ranged between 23.3 and 23.8°C. One-way ANOVA revealed a significant effect of treatment on baseline $T_{Skin}$ ($F_{(3, 31)}$ = 3.691, $p$ = 0.0221, $\eta^2$ = 0.2632; Figure 6C), with *post hoc* analyses showing that only $E_2$-treated mice had significantly lower baseline $T_{Skin}$ compared to vehicle-treated mice ($p < 0.05$).

Collectively, our results indicate that a relatively low dose of EGX358 reduced transient increases in tail skin vasodilation induced by neurokinin B signaling to similar extents as $E_2$ and DPN. However, only $E_2$, but not ERβ agonists alone, reduced baseline $T_{Skin}$ when compared to vehicle treatment.

*EGX358 and Other Estrogenic Compounds did not Affect Anxiety-like or Locomotor Behaviors*

Long-term OVX in rats and mice is associated with increased anxiety-like behaviors when compared to sham-operated controls, and these behaviors are reduced by systemic administration of estrogenic compounds, including $E_2$ and DPN, relative to vehicle treatment (Frye and Walf, 2004; Lagunas et al., 2010; Li et al., 2014; Lund et al., 2005; Oyola et al., 2012; Walf and Frye, 2006, 2005b). Therefore, we next sought to

determine the effects of EGX358 on anxiety-like behaviors. The day after completion of the senktide tail vasodilation test (day 23 of treatment), mice were gavaged with vehicle (n=10), $E_2$ (n=10), DPN (n=8), or EGX358 (n=10) (Figures 2, 3). One hour later, mice were placed into an empty OF box and the time, distance traveled, fecal boli, and entries in the outer, middle, and center zones were recorded. One-way ANOVAs revealed no significant treatment effects for fecal boli in the outer, middle, and center zones or for total fecal boli (Table 1). Similarly, total distance traveled, distance in the outer, middle, and center zones, and entries into the outer, middle, and center zones did not differ significantly among the groups (Table 1). Although the groups did not differ in time spent in the outer or middle zones, the treatment effect was significant for time spent in the center zone ($F_{(3, 33)}$ = 4.269, $p$ = 0.0118, $\eta^2$ = 0.2796; Table 1). *Post hoc* analysis revealed that $E_2$-treated mice spent significantly more time in the center zone than all other treatment groups (all $p$ values < 0.05).

We also measured time and bouts spent grooming/barbering and rearing. The main effect of treatment was significant for time spent grooming/barbering ($F$(3, 31) = 3.506, $p$ = 0.0268, $\eta^2$ = 0.2534; Table 1). Although *post hoc* tests did not indicate significant differences between any of the treatment groups, the treatment effect may have been driven by modestly reduced time grooming/barbering in $E_2$- and EGX358-treated mice relative to vehicle- and DPN-treated mice. The groups also differed significantly in time spent rearing ($F_{(3, 33)}$ = 4.278, $p$ = 0.0117, $\eta^2$ = 0.2800; Table 1), due to increased rearing in $E_2$-treated mice relative to vehicle. No significant effects of treatment were observed for bouts of grooming/barbering or rearing (Table 1).

Next, we measured the effects of long-term treatment on anxiety-like behaviors in the EPM. One day after completion of the OF test (day 25 of treatment), mice were gavaged with vehicle (n=10), $E_2$ (n=10), DPN (n=8), or EGX358 (n=9) and were placed into the EPM apparatus one hour later (Figures 2, 3). For data analysis, the EPM was divided into three zones: open arms, closed arms, and center. The time spent, number of entries, and number of fecal boli in each zone was recorded, as were total entries into all zones and total boli. However, no treatment differences were observed in any of these measures (Table 1). We also assessed "peeking" behavior by quantifying the number of peeking bouts and time spent peeking into the open arms or over the edge of the apparatus. As with the other EPM measures, no significant treatment differences were observed in either of these measurements (Table 1).

**Table 1**
Treatment group means for measures of anxiety-like behavior.

| Task | Measure | Vehicle | $E_2$ | DPN | EGX358 |
|------|---------|---------|-------|-----|--------|
| Open field | Sample size (n) | 10 | 10 | 8 | 10 |
| | Time in center (s) | *8.32 ± 1.84* | *17.95 ± 1.43\** | *8.00 ± 1.27* | *6.99 ± 0.66* |
| | Time in middle (s) | 103.80 ± 13.36 | 104.60 ± 9.43 | 84.14 ± 12.17 | 94.28 ± 12.57 |
| | Time in outer (s) | 487.90 ± 14.43 | 477.50 ± 12.55 | 507.00 ± 12.77 | 498.10 ± 12.82 |
| | Center entries | 13.90 ± 2.12 | 18.11 ± 2.35 | 12.75 ± 1.81 | 10.80 ± 1.30 |
| | Middle entries | 64.44 ± 1.97 | 72.20 ± 6.57 | 61.38 ± 7.28 | 52.40 ± 2.90 |
| | Outer entries | 49.56 ± 1.78 | 51.50 ± 3.93 | 48.88 ± 5.95 | 41.90 ±2.31 |
| | Distance in center (m) | 1.14 ± 0.18 | 1.54 ± 0.14 | 1.24 ± 0.22 | 0.98 ± 0.13 |
| | Distance in middle (m) | 11.64 ± 0.58 | 12.16 ± 1.03 | 10.68 ± 1.38 | 9.46 ± 0.59 |
| | Distance in outer (m) | 26.99 ± 1.41 | 29.43 ± 1.78 | 30.92 ± 1.62 | 31.82 ± 3.93 |
| | Total distance (m) | 40.69 ± 2.51 | 44.34 ± 2.95 | 42.84 ± 2.82 | 42.26 ± 3.91 |
| | Center boli | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | Middle boli | 0.20 ± 0.13 | 0.11 ± 0.11 | 0.14 ± 0.14 | 0.20 ± 0.13 |
| | Outer boli | 0.90 ± 0.38 | 0.22 ± 0.15 | 0.63 ± 0.26 | 0.44 ± 0.18 |
| | Total boli | 0.78 ± 0.32 | 0.33 ± 0.24 | 1.00 ± 0.38 | 0.89 ± 0.31 |
| | Bouts Barbering | 7.22 ± 0.80 | 5.70 ± 1.15 | 6.00 ± 0.93 | 5.22 ± 0.70 |
| | Time barbering (s) | *19.86 ± 2.50* | *12.82 ± 1.64* | *20.13 ± 2.86* | *13.89 ± 0.81* |
| | Bouts rearing | 98.11 ± 5.85 | 117.40 ± 5.94 | 107.00 ± 8.59 | 107.60 ± 5.17 |
| | Time rearing (s) | *108.60 ± 5.73* | *145.20 ± 6.79#* | *122.30 ± 13.05* | *135.90 ± 6.18* |
| Elevated plus maze | Sample size (n) | 10 | 10 | 8 | 10 |
| | Time in open arms (s) | 12.75 ± 3.28 | 20.59 ± 5.66 | 9.26 ± 2.94 | 11.68 ± 3.91 |
| | Time in closed arms (s) | 510.40 ± 9.62 | 468.80 ± 9.51 | 498.70 ± 7.55 | 497.20 ± 8.19 |
| | Time in center (s) | 79.36 ± 4.74 | 84.87 ± 4.70 | 84.63 ± 6.98 | 94.56 ± 6.95 |
| | Open arm entries | 1.80 ± 0.47 | 3.30 ± 7.61 | 1.43 ± 0.57 | 2.33 ± 0.69 |
| | Closed arm entries | 23.80 ± 1.07 | 24.60 ± 1.44 | 27.38 ± 1.31 | 28.67 ± 1.92 |
| | Center entries | 25.30 ± 1.45 | 27.60 ± 1.72 | 28.88 ± 1.27 | 30.22 ± 1.89 |
| | Total entries | 50.90 ± 2.78 | 55.50 ± 3.26 | 58.38 ± 2.54 | 61.22 ± 3.84 |
| | Open arms boli | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | Closed arms boli | 0.00 ± 0.00 | 0.11 ± 0.11 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | Center boli | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | Total boli | 0.00 ± 0.00 | 0.11 ± 0.11 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | Peeking bouts | 18.56 ± 1.41 | 19.11 ± 1.17 | 19.38 ± 1.32 | 21.56 ± 1.77 |
| | Peeking time (s) | 38.43 ± 3.14 | 42.69 ± 3.12 | 40.88 ± 4.31 | 45.79 ± 2.63 |

Measures in which the main effects of treatment is significant are highlighted in bold and italics.
* $p < 0.05$ relative to all other groups.
# $p < 0.05$ relative to vehicle.

Together, our results also suggest a modest anxiolytic effect of long-term oral $E_2$ treatment, but not ERβ agonism, at least at the doses tested, in young ovariectomized mice. Additionally, our results suggest no effect of $E_2$ treatment or ERβ agonism on locomotor behaviors.

*EGX358 and Other Estrogenic Compounds did not Affect Depression-Like Behaviors*

As with anxiety-like behaviors, previous research indicates that OVX mice and rats display greater depression-like behaviors when compared to sham-operated controls, and that these behaviors are reduced by administration of estrogenic compounds, including $E_2$ and DPN, compared to vehicle treatment (Li et al., 2014; Rocha et al., 2005; Walf et al., 2004; Walf & Frye, 2006, 2007). Therefore, we next examined potential effects of long-term EGX358 treatment on depression-like behaviors in the TST and FST. One day following completion of EPM testing (day 27 of treatment), mice were gavaged with vehicle (n=10), $E_2$ (n=10), DPN (n=8), or EGX358 (n=10) and were suspended by their tails one hour later (Figures 2, 3). The time spent immobile, latency to first bout of immobility, and number of fecal boli produced were recorded. One-way ANOVAs revealed no significant treatment effects on any measure (Table 2). The next day (day 30 of treatment), mice were gavaged and placed into the FST cylinder one hour later. As in the TST, time spent immobile, latency to first bout of immobility, and number of fecal boli were recorded. Similar to the TST, no treatment effects were significant for any measure (Table 2). Collectively, these findings suggest that long-term EGX358 treatment at the current dose does not affect depression-like behaviors in a mouse model of menopause.

These findings suggest no effect of long-term oral $E_2$ treatment or ERβ agonism, at least at the doses tested, on depression-like behaviors in young ovariectomized mice.

**Table 2**
Treatment group means for measures of depression-like behavior.

| Task | Measure | Vehicle | $E_2$ | DPN | EGX358 |
|---|---|---|---|---|---|
| Tail suspension test | Sample size (n) | 10 | 10 | 8 | 10 |
| | Time immobile (s) | $212.00 \pm 5.58$ | $181.00 \pm 12.91$ | $199.30 \pm 6.92$ | $181.90 \pm 11.10$ |
| | Latency to first immobility (s) | $8.90 \pm 1.89$ | $9.07 \pm 1.78$ | $12.58 \pm 4.43$ | $5.84 \pm 1.21$ |
| | Total boli | $2.50 \pm 0.79$ | $2.10 \pm 0.53$ | $2.38 \pm 0.60$ | $0.89 \pm 0.39$ |
| Forced swim test | Sample size (n) | 10 | 10 | 8 | 10 |
| | Time immobile (s) | $85.33 \pm 10.45$ | $98.03 \pm 17.38$ | $82.88 \pm 10.86$ | $91.31 \pm 14.55$ |
| | Latency to first immobility (s) | $3.45 \pm 1.10$ | $1.53 \pm 0.44$ | $2.75 \pm 1.18$ | $6.59 \pm 2.04$ |
| | Total boli | $4.56 \pm 0.50$ | $4.67 \pm 0.37$ | $4.00 \pm 0.54$ | $3.56 \pm 0.38$ |

*EGX358 Enhanced Both Spatial and Object Recognition Memory*

Our laboratory has previously demonstrated that a single post-training intraperitoneal (i.p.) injection of $E_2$ enhances spatial memory consolidation in the Morris water maze and recognition memory consolidation in OR (Gresack & Frick, 2006b), and that a single post-training administration (i.p. injection or oral gavage) of DPN or EGX358 enhances memory consolidation in both OR and OP in young ovariectomized mice (Hanson et al., 2018). Here, we aimed to determine whether long-term daily gavage of these compounds at the same doses would enhance memory in the OP and OR tasks. OP training and testing began on day 41 of treatment and OR training and testing started on day 52 of treatment (Figures 2, 3).

One hour prior to OP training, mice were gavaged with vehicle (n=10), $E_2$ (n=9), DPN (n=7), or EGX358 (n=8) (Figure 3). Twenty-three hours later, mice were again gavaged with their respective treatments and tested one hour later. Learning within a group was demonstrated with one-sample *t*-tests, which revealed that mice receiving $E_2$ ($t_{(8)}$ = 2.320, *p* = 0.0489, *d* = 0.7728; Figure 7A), DPN ($t_{(6)}$ = 4.266, *p* = 0.0053, *d* = 1.6138), or EGX358 ($t_{(7)}$ = 3.117, *p* = 0.0169, *d* = 1.1026) spent significantly more time than chance (15 s) with the moved object, whereas vehicle-treated mice did not ($t_{(9)}$ = 1.654, *p* = 0.1326). Although a one-way ANOVA revealed no main effect of treatment ($F_{(3, 30)}$ = 1.710,

$p = 0.186$), the pattern of within-group learning suggests that long-term treatment with $E_2$, DPN, or EGX358 enhanced spatial memory formation in the OP task.

OR was conducted similarly, except that the delay between training and testing was 48 hours instead of 24 hours, requiring mice to be gavaged with vehicle (n=9), $E_2$ (n=9), DPN (n=7), or EGX358 (n=10) three times: one hour prior to training, and again twenty-three and forty-seven hours later (Figure 3). Mice were tested one hour after the third gavage. One-sample $t$-tests revealed that whereas $E_2$- ($t_{(8)} = 2.439$, $p = 0.0406$, $d = 0.8128$) and EGX358-treated ($t_{(9)} = 4.342$, $p = 0.0019$, $d = 1.3732$; Figure 7B) mice spent more time than chance with the novel object, vehicle- ($t_{(8)} = 1.045$, $p = 0.3267$) and DPN-treated ($t_{(6)} = 2.400$, $p = 0.0533$) mice did not. Although the DPN group mean was similar to that for EGX358, its variability was higher, likely due to its smaller sample size. Again, the main effect of treatment was not significant ($F_{(3,}$
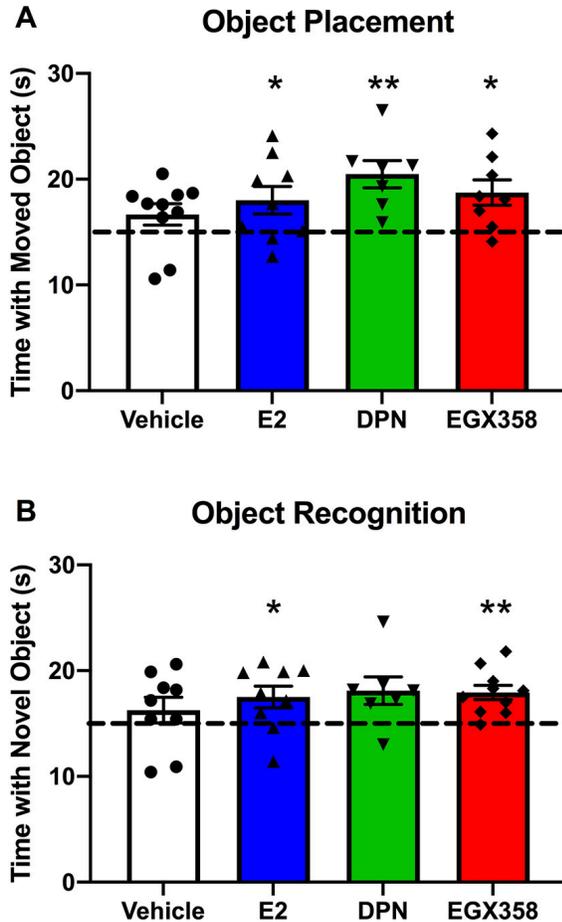
Figure 7. Long-term treatment with EGX358 enhanced spatial and object recognition memory.

**Figure 7. Long-term treatment with EGX358 enhanced spatial and object recognition memory**. Following completion of forced swim testing, mice continued daily gavage of vehicle (n=10), $E_2$ (n=9), DPN (n=8), or EGX358 (n=10) without being tested for 7-8 days, after which they began training and testing in OP and, subsequently, OR. A) Mice were gavaged 1 hour prior to training and testing phases of the OP task. Mice treated with $E_2$, DPN, or EGX358, but not vehicle, spent significantly more time than chance (dashed line at 15 s) with the moved object during the testing phase of OP. B) Similar to OP, mice gavaged with $E_2$ or EGX358 1 hour prior to training, 24 hours later, and 1 hour prior to testing spent significantly more time than chance with the novel object during the testing phase of OR. $E_2$, 17$\beta$-estradiol; DPN, diarylpropionitrile; OP, object placement; OR, object recognition. Error bars represent mean ± SEM. *$p < 0.05$, compared to chance.

$_{31)}$ = 0.6365, $p$ = 0.5972), but the within-subjects analyses suggest that long-term $E_2$ or EGX358 treatment enhanced object recognition memory.

Together, these findings suggest that long-term oral gavage of $E_2$ and EGX358 enhances spatial and object recognition memory in young ovariectomized mice, with DPN providing some similar benefits.

*Long-Term EGX358 Treatment did not Affect Protein Activation or Expression in the DH, mPFC, Amygdala, or Hypothalamus*

Given our physiological and behavioral results demonstrating that long-term oral treatment with $E_2$, DPN, and EGX358 reduce drug-induced increases in vasodilation and enhance memory, and that $E_2$ treatment modestly alleviates anxiety-like behaviors, we next wished to examine the molecular effects of these treatments in ERβ-laden brain regions implicated in memory, affective symptoms, and hot flashes. Therefore, the day after the final tail skin temperature measurement, mice were gavaged with vehicle (n=10), E2 (n=9), DPN (n=8), or EGX358 (n=10) and euthanized 30 min later (Figure 4). Tissues from the DH, mPFC, amygdala, and hypothalamus were collected rapidly on wet ice for subsequent Western blot analyses.

We and others have demonstrated repeatedly that acute or long-term 17β-estradiol and DPN treatment promotes cell signaling, the activation of plasticity-mediating kinases and trophic factors, such as the p42 isoform of ERK and BDNF, and increases in dendritic spine densities in the DH in rodents, which are associated with better memory outcomes (Boulware et al., 2013; Fernandez et al., 2008; Inagaki et al., 2012; Jain et al., 2019; Y. Zhao et al., 2017). Although less is known about the actions of estrogenic signaling in the mPFC, similar activation and spine density changes are seen therein following acute or

long-term treatment with $E_2$ and phytoestrogens (Inagaki et al., 2012; Pan et al., 1999; Zeidan et al., 2011). Furthermore, estrogenic signaling appears to reduce activity in both the amygdala and median preoptic area of the hypothalamus, which appear to mediate the anxiolytic, anti-depressive, and vasodilation-reducing effects of estrogens (Dacks et al., 2011; Zeidan et al., 2011). Therefore, regional homogenates were blotted for phospo-p42 and phospho-p44 ERK, phospho-CaMKIIα, phospho-cofilin, the ratio of GluA1 to GluA2/3/4, and/or the total expressions of F-actin, PSD95, SNAP25, c-Fos, synaptophysin, BDNF, and ER$\beta$ relative to $\beta$-actin expression, depending on the region being analyzed. Specifically, we hypothesized that in regions highly implicated in learning and memory outcomes, namely the DH and mPFC, long-term treatment with $E_2$, DPN, and EGX358 would increase markers of cellular activity (c-Fos), phosphorylation of rapid kinases critical for learning and memory outcomes (p42 ERK and CaMKIIα), trophic factors critical for learning and memory outcomes (BDNF), presynaptic markers of vesicular release of neurotransmitters (SNAP25, synaptophysin), postsynaptic markers of plasticity (PSD95, GluA ratio), and postsynaptic markers of actin remodeling (F-actin, phospho-cofilin), and prevent a decline in sensitivity to treatment (ER$\beta$) to reflect memory outcomes. In the amygdala, a region highly associated with affective outcome, we hypothesized $E_2$, DPN, and EGX358 treatment would lower expression of c-Fos, phospho-p42 ERK, BDNF, synaptophysin, PSD95, and F-actin, reflecting our anxiety-like measurements. Finally, given that all three treatments reduced drug-induced vasomotor symptoms relative to vehicle, and $E_2$ prevented a rise in baseline $T_{Skin}$, we hypothesized that they would reduce c-Fos expression in the hypothalamus compared to vehicle treatment.

Surprisingly, and in contrast to their memory-enhancing effects, E2, DPN, and
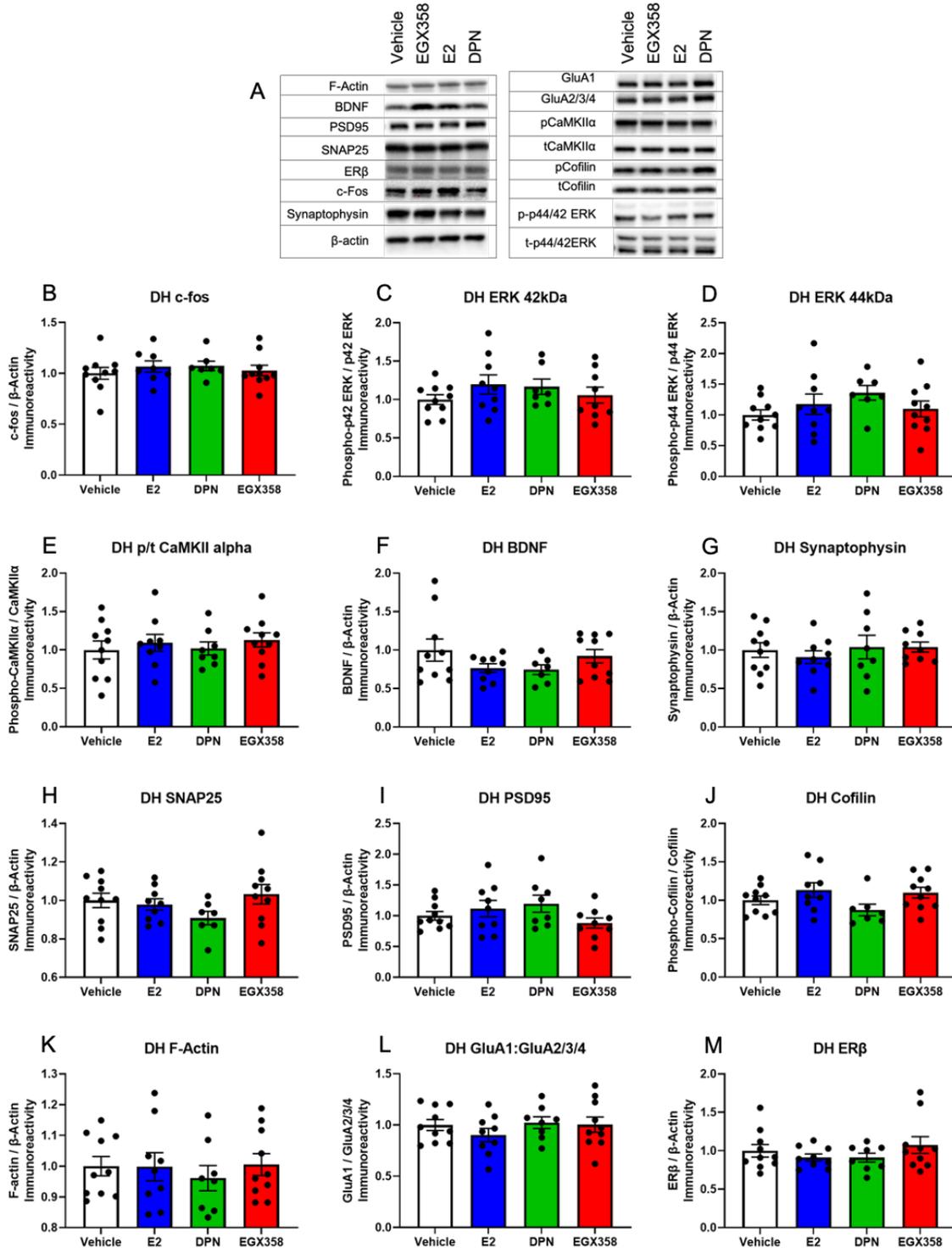


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EGX358 did not alter protein expression or phosphorylation of any target proteins in the

DH (Figure 8A). Namely, there were no significant main effects of treatment in the DH on

**Figure 8. Long-term EGX358 treatment does not affect protein expression or phosphorylation in the dorsal hippocampus.** The day following completion of all behavioral and physiological measurements, mice were gavaged with vehicle (n=10), $E_2$ (n=9), DPN (n=8), or EGX358 (n=10). Mice were euthanized 30 min later, and DH tissue was collected for Western blot analysis. A) Representative images proteins blotted. No treatment effects were found in expressions of B) c-Fos, F) BDNF, G) synaptophysin, H) SNAP25, I) PSD95, or K) F-actin, phosphorylation levels of C) 42kDa ERK, D) 44kDa ERK, E) CaMKIIα, or J) cofilin, or L) the ratio of GluA1:GluA2/3/4. M) Additionally, there were no differences in ERβ expression due to treatment. DH, dorsal hippocampus; ERK, extracellular signal-regulated kinase; CaMKII, calcium/calmodulin-dependent kinase II; BDNF, brain-derived neurotrophic factor; PSD95, postsynaptic density protein 95; F-actin, filamentous actin; GluA, AMPA receptor subunit; ERβ, estrogen receptor β; $E_2$, 17β-estradiol; DPN, diarylpropionitrile. Error bars represent mean $\pm$ SEM. All $p > 0.05$.

the phosphorylation of the 42kDa ($F_{(3, 31)}$ = 0.8945, $p$ = 0.4550, Figure 8C) or 44kDa isoforms of ERK ($F_{(3, 32)}$ = 1.240, $p$ = 0.2967, Figure 8D), CaMKIIα ($F_{(3, 33)}$ = 0.3678, $p$ = 0.7767, Figure 8E), or cofilin ($F_{(3, 32)}$ = 2.126, $p$ = 0.1151, Figure 8J), the expressions of c-Fos ($F_{(3, 31)}$ = 0.3950, $p$ = 0.7575, Figure 8B), BDNF ($F_{(3, 32)}$ = 1.444, $p$ = 0.2483, Figure 8F), synaptophysin ($F_{(3, 32)}$ = 0.3665, $p$ = 0.7776 Figure 8G), SNAP25 ($F_{(3, 32)}$ = 1.485, $p$ = 0.2373, Figure 8H), PSD95 ($F_{(3, 32)}$ = 1.572, $p$ = 0.2154, Figure 8I), or F-actin ($F_{(3, 33)}$ = 0.2525, $p$ = 0.8589, Figure 8K), or the ratio of GluA1:GluA2/3/4 ($F_{(3, 33)}$ = 0.6963, $p$ = 0.5610, Figure 8L). Importantly, these results cannot be accounted for by the alteration of ERβ levels, as there were no differences in ERβ expression among treatment groups here ($F_{(3, 33)}$ = 0.9581, $p$ = 0.4240, Figure 8M).

As in the DH, surprisingly, there were no treatment effects on protein expression or phosphorylation in the mPFC (Figure 9A). Namely, there were no significant treatment effects in the mPFC on the phosphorylation of the 42kDa ($F_{(3, 32)}$ = 0.5117, $p$ = 0.6771, Figure 9C) or 44kDa isoforms of ERK ($F_{(3, 32)}$ = 1.191, $p$ = 0.3289, Figure 9D), CaMKIIα ($F_{(3, 30)}$ = 0.9709, $p$ = 0.4194, Figure 9E), or cofilin ($F_{(3, 31)}$ = 1.223, $p$ = 0.3179, Figure 9J), the expressions of c-Fos ($F_{(3, 30)}$ = 0.1704, $p$ = 0.9155, Figure 9B), BDNF ($F_{(3, 30)}$ = 0.7244, $p$ = 0.5454, Figure 9F), synaptophysin ($F_{(3, 32)}$ = 0.3573, $p$ = 0.7842, Figure 9G), SNAP25 ($F_{(3, 31)}$ = 0.8423, $p$ = 0.4812, Figure 9H), PSD95 ($F_{(3, 31)}$ = 1.063, $p$ = 0.3791, Figure 9I),
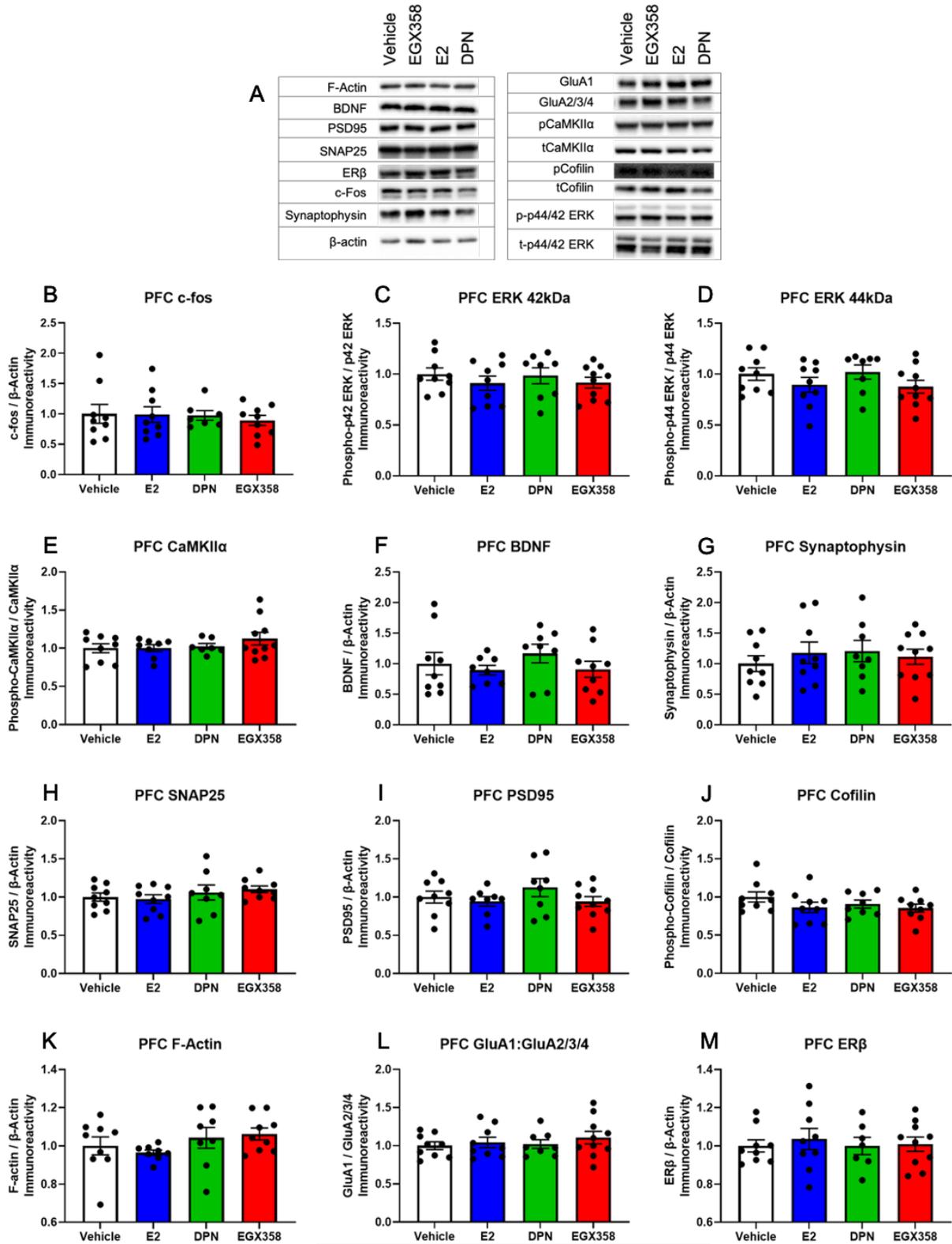
Figure 9. Figure legend on next page.

or F-actin ($F_{(3, 30)}$ = 1.190, $p$ = 0.3301, Figure 9K), or the ratio of GluA1:GluA2/3/4 ($F_{(3, 32)}$

**Figure 9. Long-term EGX358 treatment does not affect protein expression or phosphorylation in the medial prefrontal cortex.** The day following completion of all behavioral and physiological measurements, mice were gavaged with vehicle (n=9), $E_2$ (n=9), DPN (n=8), or EGX358 (n=10). Mice were euthanized 30 min later, and mPFC tissue was collected for Western blot analysis. A) Representative images proteins blotted. No treatment effects were found in expressions of B) c-Fos, F) BDNF, G) synaptophysin, H) SNAP25, I) PSD95, or K) F-actin, phosphorylation levels of C) 42kDa ERK, D) 44kDa ERK, E) CaMKII$\alpha$, or J) cofilin, or L) the ratio of GluA1:GluA2/3/4. M) Additionally, there were no differences in ER$\beta$ expression due to treatment. PFC, prefrontal cortex; ERK, extracellular signal-regulated kinase; CaMKII, calcium/calmodulin-dependent kinase II; BDNF, brain-derived neurotrophic factor; PSD95, postsynaptic density protein 95; F-actin, filamentous actin; GluA, AMPA receptor subunit; ER$\beta$, estrogen receptor $\beta$; $E_2$, 17$\beta$-estradiol; DPN, diarylpropionitrile. Error bars represent mean $\pm$ SEM. All $p > 0.05$.

= 0.4939, $p$ = 0.6890, Figure 9L). Similar to the DH, these findings cannot be explained by differences in ER$\beta$ levels, as there were no treatment effects on ER$\beta$ expression ($F_{(3, 31)}$ = 0.1581, $p$ = 0.9236, Figure 9M).

Perhaps unsurprisingly, given the modest anxiety- and depression-like behavioral results, long-term treatment with $E_2$, DPN, or EGX358 did not alter protein expression or phosphorylation in the amygdala (Figure 10A). Namely, there were no treatment effects in the amygdala on the phosphorylation of the 42kDa ($F_{(3, 32)}$ = 0.4723), $p$ = 0.7037, Figure 10C) or 44kDa isoforms of ERK ($F_{(3, 32)}$ = 0.8206, $p$ = 0.4921, Figure 10D) or the expressions of c-Fos ($F_{(3, 31)}$ = 0.6774, $p$ = 0.5725, Figure 10B), BDNF ($F_{(3, 32)}$ = 0.9996, $p$ = 0.4057, Figure 10E), synaptophysin ($F_{(3, 31)}$ = 1.461, $p$ = 0.2443, Figure 10F), PSD95 ($F_{(3, 33)}$ = 0.4471, $p$ = 0.7210, Figure 10G), or F-actin ($F_{(3, 32)}$ = 0.9908, $p$ = 0.4096, Figure 10H).

Finally, given the reduction in drug-induced vasodilation due to estrogenic treatment and the lower baseline temperature noted after 63 days of $E_2$ treatment relative to vehicle treatment, it was also surprising to find no treatment effects on c-Fos expression in the hypothalamus ($F_{(3, 33)}$ = 0.07415, $p$ = 0.9735, Figure 11A, B).
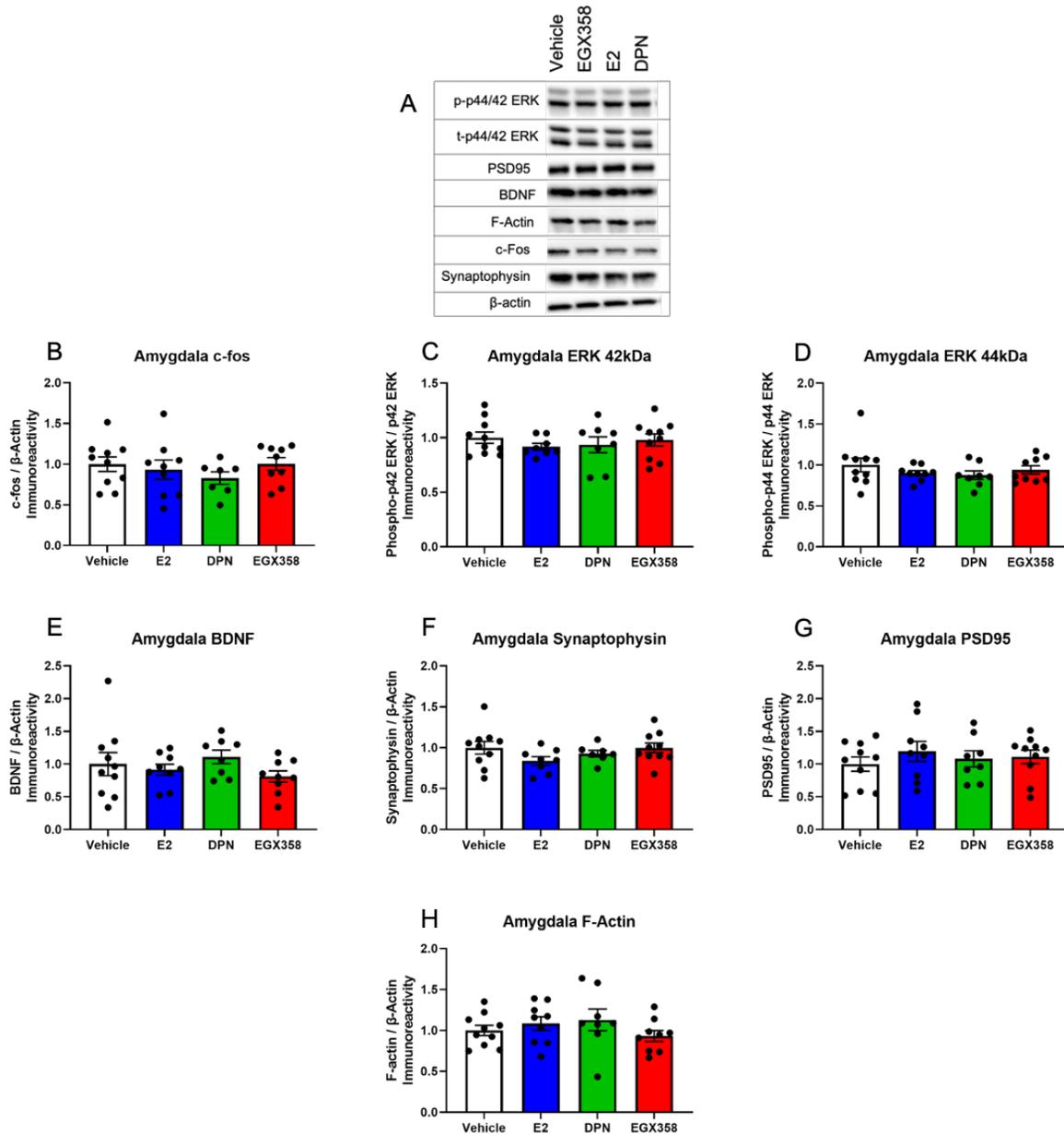
**Figure 10. Long-term EGX358 treatment does not affect protein expression or phosphorylation in the amygdala.** The day following completion of all behavioral and physiological measurements, mice were gavaged with vehicle (n=10), $E_2$ (n=9), DPN (n=8), or EGX358 (n=10). Mice were euthanized 30 min later, and amygdala tissue was collected for Western blot analysis. A) Representative images proteins blotted. No treatment effects were found in expressions of B) c-Fos, E) BDNF, F) synaptophysin, G) PSD95, or H) F-actin or phosphorylation levels of C) 42kDa ERK, D) 44kDa ERK. ERK, extracellular signal-regulated kinase; BDNF, brain-derived neurotrophic factor; PSD95, postsynaptic density protein 95; F-actin, filamentous actin; $E_2$, 17β-estradiol; DPN, diarylpropionitrile. Error bars represent mean $\pm$ SEM. All $p > 0.05$.

## Discussion

We and others have previously demonstrated roles for ERβ signaling in enhancing memory processes, reducing anxiety- and depression-like behaviors, and reducing vasomotor symptoms in OVX rodents (Boulware et al., 2013; Hanson et al., 2018; Jacome et al., 2010; J. Kim & Frick, 2017; Li et al., 2014; Lund et al., 2005; Opas et al., 2006; Oyola et al., 2012; Pereira et al., 2014; Rocha et al., 2005; Walf et al., 2004, 2006, 2008; Walf & Frye, 2005; L. Zhao et al., 2011). However, the present study is the first to examine the effects of long-term oral ERβ agonist treatment on preclinical indices of menopausal symptoms in OVX mice. This work is particularly notable for its evaluation of EGX358, which is 750-fold more selective for ERβ over ERα and is therefore the most selective ERβ synthetic agonist available to date (Hanson et al., 2018). Here, we showed that daily gavage of EGX358, DPN, and E$_2$ reduced senktide-induced increases in T$_{Skin}$ after ~2.5 weeks of treatment, and 9 weeks of E$_2$, but not ERβ agonist alone, treatment reduced T$_{Skin}$ compared to vehicle treatment. Next, we demonstrated that, although EGX358, DPN, and E$_2$ had largely no effects on anxiety- or depression-like behaviors, daily gavage for 8-9 weeks enhanced spatial and object recognition memory in the OP and OR tasks. Surprisingly, there were no effects of 64 days of E2, DPN, or EGX358 treatment on



**Figure 10. Long-term EGX358 treatment does not affect c-Fos in the hypothalamus.** The day following completion of all behavioral and physiological measurements, mice were gavaged with vehicle (n=10), E$_2$ (n=9), DPN (n=8), or EGX358 (n=10). Mice were euthanized 30 min later, and hypothalamic tissue was collected for Western blot analysis. A) Representative images proteins blotted. B) No treatment effects were found in expression of c-Fos. E$_2$, 17β-estradiol; DPN, diarylpropionitrile. Error bars represent mean ± SEM. $p > 0.05$.

cellular activity- and synaptic plasticity-related protein expression or phosphorylation in the DH, mPFC, amygdala, or hypothalamus, regions which are all highly implicated in learning and memory outcomes, affective symptom presentation, and hot flash mediation. Importantly, long-term oral administration of EGX358 did not result in premature death or adverse health effects in any mice, although estrogenic treatment also failed to prevent weight gain compared to vehicle. Collectively, these results provide promising evidence that long-term administration of selective ERβ agonists like EGX358 may be effective treatment options for some menopausal symptoms, although the cellular and molecular mechanisms underlying EGX358's behavioral and physiological effects remain to be determined.

*Effects on Vasodilation*

Our data showing that both DPN and our novel, highly selective ERβ agonist, EGX358, prevent senktide-mediated increases in $T_{Skin}$ are the first to demonstrate a role for ERβ activation in this model. Previous findings have demonstrated that systemic senktide administration produces rapid, transient increases in $T_{Skin}$ in male and gonadally-intact and OVX female mice (Krajewski-Hall et al., 2018; Krull et al., 2017). These effects are mediated by the binding of senktide to tachykinin 3 receptors in the median preoptic area of the hypothalamus, thereby activating warm-sensitive neurons in this region and initiating vasodilation and cold-seeking behaviors in rodents (Krull et al., 2017; Mittelman-Smith et al., 2015; Padilla et al., 2018; Rance et al., 2013; Tan et al., 2016). Importantly, previous studies have also demonstrated that tachykinin 3 receptor-mediated tail vasodilation responses are reduced in intact compared to OVX female mice and following senktide administration in OVX mice treated chronically with $E_2$ via silastic capsule

compared to vehicle-treated mice (Krajewski-Hall et al., 2018; Padilla et al., 2018). We add to this growing body of literature by demonstrating that long-term treatment with ER$\beta$ agonists can mitigate senktide-induced increases in $T_{Skin}$. Importantly, $E_2$ (0.2 mg/kg), DPN (0.05 mg/kg), and EGX358 (0.5 mg/kg) all prevented senktide-mediated changes in $T_{Skin}$ compared to vehicle treatment, suggesting a protective role of ER$\beta$ signaling against neurokinin B-mediated hot flash symptoms. Although $T_{Skin}$ increased modestly and transiently after vehicle injection, it is highly unlikely that this increase influenced the effects of estrogenic compounds on $T_{Skin}$. Importantly, the $\Delta T_{Skin}$ increase following senktide injection was more than 2-fold higher than after vehicle injection, with $\Delta T_{Skin}$ of under 2$^{\circ}$C after vehicle injection and of 4-5$^{\circ}$C after senktide injection. Additionally, only senktide-treated mice exhibited characteristic tail rattles and expressed cold-seeking behaviors, such as moving cage bedding around to create bare, cool spots, where they would reside for much of the test, behaviors that were unaffected by estrogenic compound treatment. Others have made similar observations following senktide treatment in mice (Krajewski-Hall et al., 2018; Krull et al., 2017). These quantitative and qualitative differences suggest that the estrogenic compounds specifically mitigated the vasodilatory response to senktide, not a more general reaction to the injection procedure. As such, our data indicate that long-term oral treatment with $E_2$ or ER$\beta$ agonists such as EGX358 can reduce vasodilation in a neurokinin B-mediated model of hot flashes.

Interestingly, nine weeks of ER$\beta$ agonist treatment did not affect baseline $T_{Skin}$. Long-term OVX is a common method for modeling vasomotor symptoms and their treatment in rodents, as this surgical method induces gradual increases in tail vasodilation, and chronic treatment with estrogenic compounds often prevents this elevation in temperature

in both mice and rats (Berendsen et al., 2001; Bowe et al., 2006; Dacks & Rance, 2010; Kobayashi et al., 1995; Opas et al., 2004, 2006; L. Zhao et al., 2011). We recapitulate these findings by demonstrating that $E_2$ (0.2 mg/kg) administered for 9 weeks via gavage reduces baseline $T_{Skin}$ in OVX mice relative to vehicle-treated mice. However, DPN (0.05 mg/kg) and EGX358 (0.5 mg/kg) treatment did not alter $T_{Skin}$ relative to vehicle treatment at this time point. These findings contrast with previous studies demonstrating that ERβ-selective phytoestrogen diets and DPN injections can reduce increases in $T_{Skin}$ compared to vehicle treatment following OVX in both rats and mice (Bowe et al., 2006; Opas et al., 2006; L. Zhao et al., 2011). However, the long-term design of our study, in addition to the relatively low dosage of both DPN and EGX358, may have contributed to these contrasting results. For example, Bowe et al. (2006) showed that four days of subcutaneous injections of DPN at 0.6 mg/kg, a dose twelve-fold greater than that administered here, lowered $\Delta T_{Skin}$ relative to first-day measurements compared to vehicle-treated OVX rats. As our chosen EGX358 dose is proportional to our DPN dose, based upon the previously established relative potency of the compounds, a higher dose of EGX358 may have more effectively lowered $\Delta T_{Skin}$ relative to vehicle treatment (Hanson et al., 2018). Additionally, previous studies have demonstrated that $T_{Skin}$ is a circadian rhythm, and that sensitivity to treatment with estrogenic compounds may be higher during the dark-phase than the light-phase (Girbig et al., 2012; Williams et al., 2010). As our measurements were made during the light-phase and only at the end of the study, it is possible that effects of long-term EGX358 treatment on baseline $T_{Skin}$ may have been missed or obscured. Follow-up studies utilizing higher ERβ agonist doses,

testing multiple times throughout the study, and/or testing during the dark-phase may prove useful in demonstrating these effects.

*Effects on Anxiety- and Depression-Like Behaviors*

Previous research has repeatedly demonstrated an important role for ERβ activation in reducing anxiety- and depression-like behaviors in OVX mice and rats (Frye and Walf, 2004; Lagunas et al., 2010; Li et al., 2014; Lund et al., 2005; Oyola et al., 2012; Rocha et al., 2005; Walf and Frye, 2007, 2005b, 2004). Surprisingly, the present study showed only an effect of $E_2$ treatment on time in the center of and time spent rearing in the OF, but no other effects of any treatment in the OF, EPM, TST, or FST compared to vehicle. This discrepency, especially given the lack of effects of either ERβ agonist, could be due to a number of factors. Previous studies have shown that sensitivity to estrogenic compounds in these tasks is highly dependent upon dose, injection schedule, solvent used, and administration route (as reviewed in Walf & Frye, 2006). As discussed previously, we selected doses of $E_2$, DPN, and EGX358 which had previously been shown to enhance memory consolidation in the Morris water maze, OP, and OR tasks when administered acutely in OVX mice (Gresack and Frick, 2006; Hanson et al., 2018). Although 0.2 mg/kg $E_2$ should produce physiological levels of $E_2$ when administered via i.p. injection, we cannot be sure of these levels after oral gavage (Akinci & Johnston, 1997; Gresack & Frick, 2006b). Additionally, 0.05 mg/kg DPN and 0.5 mg/kg EGX358 are relatively low compared to other studies in which 0.1-1.0 mg/kg DPN produced anxiolytic and anti-depressive effects (Eid et al., 2020; Lund et al., 2005; Oyola et al., 2012). Thus, future studies will be necessary to determine whether higher doses of daily administered EGX358 or DPN reduce anxiety- and depression-like behaviors in these tasks.

Other factors to take into consideration include stress, administration route, and duration of treatment. A recent study demonstrated that daily subcutaneous injections of $E_2$ (0.04 mg/kg), DPN (0.1 mg/kg), or the commercially available ER$\alpha$ agonist PPT (0.1 mg/kg) for 47 days in OVX mice all reduce time spent immobile in the TST, but that chronic unpredictable stress reverses these effects, such that DPN and PPT both increase time spent immobile in this task (Eid et al., 2020). These data suggest that elevated stress may block or even reverse the effects of estrogenic compounds on measures of anxiety- and depression-like behaviors. Although gavage is a more effective method of ensuring accurate oral dosing than water- or food-based delivery, it is also more invasive and stressful than these other methods (Machholz et al., 2012). Therefore, the stress associated with gavage, combined with the relatively low doses used, may have prevented $E_2$, DPN, and EGX358 from influencing affective measures. Finally, ours is one of the longest-term studies examining the effects of ER$\beta$ agonist treatment on anxiety- and depression-like behaviors in OVX mice. Previous work found that 3-7 days is optimal for detecting anxiolytic and anti-depressive effects of $E_2$ in both OVX rats and mice, and most studies demonstrating anxiolytic and anti-depressive actions of DPN in OVX rodents have administered the compound acutely or for up to 7 days (Bernardi et al., 1989; Koss et al., 2004; Li et al., 2014; V. N. Luine et al., 1998; Lund et al., 2005; Oyola et al., 2012; Rocha et al., 2005; Walf et al., 2004; Walf & Frye, 2005, 2006). Thus, long-term ER$\beta$ agonist treatment may be less effective than short-term treatment in mitigating affective behaviors in OVX rodents. Future studies examining putative influences of dose, route of administration, treatment duration, and stress effects will be necessary to more fully understand the extent to which daily oral administration of

estrogenic compounds influences affective behaviors. Importantly, however, none of our treatments *increased* anxiety- and depression-like behaviors in any of our tasks, suggesting that anxiety- or depression-like behaviors are not side effects of long-term $E_2$, DPN, or EGX358 treatment.

*Effects on Memory*

Our results demonstrating that long-term oral EGX358 administration in OVX mice promotes spatial and object recognition memory formation in the OP and OR tasks fits well with previous results from our lab and others showing memory-enhancing effects of acute post-training ERβ agonism (Boulware et al., 2013; Hanson et al., 2018; Jacome et al., 2010; Pereira et al., 2014; Said et al., 2018; Walf et al., 2008; L. Zhao et al., 2011). Whereas many previous studies have demonstrated that a single post-training delivery of DPN either systemically or directly into the hippocampus enhances OP and OR memory consolidation in young OVX rats and mice, and that 2 days of pre-training systemic injections recapitulates these effects, the current study is one of few to demonstrate that long-term administration of ERβ agonists enhance memory (Boulware et al., 2013; Hanson et al., 2018; Jacome et al., 2010; Pereira et al., 2014; Walf et al., 2008). Previously, Zhao et al. (2011) demonstrated that treating OVX mice with an ERβ-selective phytoestrogen diet for 9 months enhanced spatial working memory in the Y-maze, and Said et al. (2018) reported that diet-based DPN treatment (~3.0 mg/kg/day) for 22 months enhanced spatial memory in the Barnes maze in OVX mice. Thus, the fact that EGX358, and to a lesser extent DPN, enhanced memory in the present study is consistent with previous reports of long-term ERβ agonist treatment.

We previously showed that acute DPN or EGX358 treatment given immediately post-training enhances OP and OR memory consolidation in young OVX mice (Hanson et al., 2018). These effects were observed after bilateral dorsal hippocampal infusion (DPN: 10 pg; EGX358: 100 pg and 1 ng), or systemic administration via i.p. injection or oral gavage (DPN: 0.05 mg/kg; EGX358: 0.5 mg/kg or 5 mg/kg) (Hanson et al., 2018). The present study adds to these findings by showing that long-term oral treatment of either DPN or EGX358 via gavage enhances spatial and object recognition memory in OVX mice. Although DPN treatment did not significantly enhance OR consolidation, there was a trend for such an effect that may have been revealed with an increased sample size.

Although this study was designed to determine effects of long-term treatment on memory and other factors, it is important to note that our effects on memory could be due not only to long-term treatment, but also to acute effects of treatment given one hour prior to training and testing. Given our study design, we cannot necessarily exclude acute effects in the improvements seen here. However, it is noteworthy that daily oral treatment with DPN or EGX358 enhanced memory in these tasks to a similar extent as our previous acute studies (Hanson et al., 2018). Namely, here, we demonstrate that long-term treatment with DPN or EGX358 promoted time spent with the moved object in OP, such that DPN treatment and EGX358 treatment resulted in 36.6% and 24.9% more time with the moved object than chance. Additionally, long-term treatment increased time with the novel object in the OR task, such that DPN treatment and EGX358 treatment resulted in 20.7% and 19.6% more time spent investigating the novel object than chance. These findings are similar to our previous report, showing that acute gavage treatment of DPN and EGX358 resulted in 29.2% and 31.5% more time with the moved object in the OP

task, and 30.7% and 29.9% more time with the novel object in the OR task (Hanson et al., 2018). It is interesting that there appears to be a slightly weaker effect of long-term treatment compared to acute treatment, although this could be due to a number of factors, including treatment timing, stress of repeated gavage, or time of testing relative to OVX surgery (1-2 weeks post-surgery in the acute study (Hanson et al., 2018) as compared to ~10 weeks, here). Although we have not tested effects of acute treatment on anxiety-like, depression-like, or vasomotor symptoms, we would expect similarly slight differences between acute and long-term administration in each of these measurements. Despite the minor difference between effect sizes of acute and long-term treatments, our finding that long-term oral treatment with our highly selective ER$\beta$ agonist, EGX358, enhanced memory consolidation in both OP and OR further lends support for its continued development as an ER$\beta$-selective HT for menopause-related memory dysfunction.

*Effects on Protein Expression and Phosphorylation*

Surprisingly, long-term administration of E$_2$, DPN, or EGX358 did not affect expression or phosphorylation of protein markers of cellular activity or synaptic plasticity in the DH, mPFC, amygdala, or hypothalamus. In particular, lack of protein or phosphorylation alterations in the DH, mPFC, and hypothalamus was unexpected, given that E$_2$, DPN, and EGX358 all reduced drug-induced vasodilation relative to vehicle and enhanced memory relative to chance. It was also expected that E$_2$ would alter protein expression and phosphorylation in the amygdala to reflect the modest anxiolytic effect treatment had in the open field task. Yet, remarkably, no proteins or phosphorylation states were altered due to treatment in any of the brain regions examined.

We investigated several proteins associated with cellular activity and synaptic plasticity events. c-Fos, an immediate early gene, is often examined as a marker of neural activity, as stimulation of a cell upregulates c-Fos expression, which functions to promote subsequent waves of transcription (Gallo et al., 2018; Tyssowski et al., 2018). Upon cellular stimulation and subsequent phosphorylation, kinases such as ERK and CaMKIIα phosphorylate hundreds of other protein targets to alter their levels of activity or, in the case of ERK, translocate into the cellular nucleus to upregulate protein expression, both processes which are directly implicated in the generation and maintenance of synaptic plasticity (Adams & Sweatt, 2002; Lisman et al., 2002; Plotnikov et al., 2019; Tyssowski et al., 2018). The neurotrophin, BDNF, is a well-characterized marker of synaptic plasticity, functioning to regulate NMDA receptor trafficking and maintenance, as well as developing and stabilizing dendritic spines, especially as has been shown in regions such as the hippocampus (Miranda et al., 2019). Synaptophysin and SNAP25 are both proteins involved in the trafficking of vesicles to the presynaptic membrane, allowing for neurotransmitter exocytosis (Tomasoni et al., 2013). Upon sufficient postsynaptic stimulation, PSD95 is recruited to the dendritic spines to anchor factors such as AMPA and NMDA receptors to the postsynaptic density, sensitizing the postsynaptic cell to future stimulation (Chen et al., 2011). To facilitate the recruitment of PSD95 and synaptic strengthening, actin is reorganized to drive changes in dendritic spines. Phosphorylation of cofilin, for example, allows for the polymerization of actin into filamentous structures (F-actin), stabilizing the synapse (Basu & Lamprecht, 2018; Gu et al., 2010). Furthermore, shuttling of GluA2/3-containing AMPA receptors into synapses is thought to gradually replace GluA1-containing AMPA receptors to stabilize plastic changes and maintain

connectivity between pre- and postsynaptic sites, which is accomplished by actin remodeling and stabilization (Basu & Lamprecht, 2018; Diering & Huganir, 2018). All of these factors work at different phases of plasticity following the activation of cells to increase the connectivity between pre- and postsynaptic domains. Therefore, these were candidate proteins to examine in regions implicated in learning, affective, and vasomotor outcomes following long-term administration of EGX358.

Given the roles $E_2$ plays as a neuromodulator in the hippocampus by inducing ERK, PKA, and PKC signaling, the trafficking and phosphorylation of AMPA and NMDA receptors, and remodeling of dendritic spine densities and arbors through the cofilin pathway, it is surprising not to have captured any similar effects in the DH due to long-term treatment with not only $E_2$, but also DPN or EGX358 (as reviewed in Taxier et al., 2020). Similar results have been show in the mPFC, where $E_2$ treatment appears to increase cellular activity and promote dendritic spine densities (Tuscher et al., 2019; Zeidan et al., 2011). Within the amygdala and hypothalamus, the opposite appears to be true, such that $E_2$ appears to downregulate activity (Dacks et al., 2011; Zeidan et al., 2011). Although much less is known about molecular function of ERβ in the mPFC, amygdala, and hypothalamus in relation, it is nonetheless surprising to not have found any protein or phosphorylation changes in any of these regions either, especially given the behavioral and physiological results of this study.

Two potential reasons for the seeming lack of biochemical results may be due to the timing and dosing of the current study. Although the majority of studies examining the molecular mechanisms underlying $E_2$ and ERβ actions in the hippocampus have utilized short-term or acute administration methods, a handful of long-term studies have

administered either DPN or utilized ERβ-selective phytoestrogen diets. In one study, feeding OVX mice a phytoestrogen diet with 83-fold selectivity for ERβ over ERα at a dose of roughly 10 mg/kg for 9 months enhanced memory outcomes on the Y-maze and promoted hippocampal BDNF and PSD95 levels (Zhao et al., 2011). Zhao et al. (2011) treated their mice for considerably longer than the current study, and at a much higher dose than that of our DPN or EGX358 groups, which could have promoted responses in the DH more readily than the current study's treatments. Another study utilizing high-phytoestrogen diets (810 μg/g) for 9 weeks showed increased dendritic spine densities in the mPFC and DH of OVX rats compared to rats given a low-phytoestrogen diet (Luine et al., 2006). The timing used by Luine et al. (2006) is remarkably close to the current study. Importantly, their phytoestrogen dose of 810 μg/g, or 810 mg/kg, is only slightly higher than that used in our standard diet, which ranges between 350 and 650 mg/kg, suggesting that diet may in our study may have obfuscated increases in plasticity that might have otherwise been seen in mice treated with low-phytoestrogen diets. Sárvári and colleagues (2016) administered DPN via osmotic pump for 29 days at a dose of 0.02 mg/animal/day in 13-month-old OVX rats showed many altered genes in the hippocampus due to treatment, most notably the increased production of mGluR1, mGluR5, GluA2, GluA3 mRNA expression, suggestive of increased sensitivity to glutamatergic signaling in this region. Although the treatment timeline was shorter than the current study, the use of aged rats and continuous delivery via osmotic pump may have led to different timing profiles of ERβ activation and in a system too dissimilar to the young mice used in the current study to see similar results. Additionally, 0.02 mg DPN/day

is roughly 13 times greater than the DPN dose utilized in the current study, which may also have affected gene and protein expression outcomes differently than here.

Importantly, another factor to consider is the timepoint at which tissues were collected following the final gavage treatment. Previous studies have shown that serotonin and metabolites of dopamine and norepinephrine are elevated in the mPFC of OVX rats 30 min following peripheral $E_2$ injection (Inagaki et al., 2010). Furthermore, dendritic spine densities in both the mPFC and DH are increased 30 min following $E_2$ injection in OVX rats (Inagaki et al., 2012). On account of these findings, in the current study, we collected DH, mPFC, amygdala, and hypothalamic tissues 30 minutes following gavage on the final day of treatment. However, the temporal dynamics of gavage may differ from that of subcutaneous injection, and to date, there are no studies examining the time it takes $E_2$ or DPN to reach the brain through gavage. Additionally, we have not conducted a time course study of EGX358 and therefore do not know how long it takes the compound to reach brain areas of interest. Therefore, it is possible we simply missed the timepoints at which $E_2$, DPN, or EGX358 may have affected protein expression and phosphorylation relative to vehicle treatment.

Another potential explanation for the lack of molecular effects could be the loss of sensitivity to treatment. For example, given that cellular activity markers like c-Fos are upregulated in the hippocampus specifically in response to novel contexts or stimulation types, it is possible that repeated gavage of $E_2$, DPN, or EGX358 was unable to generate a "novel" enough signal compared to vehicle treatment after 64 days (Bernstein et al., 2019; Bertaina-Anglade et al., 2000; Mahringer et al., 2019). Specifically, exposure to novel or semi-novel objects, contexts, or animals is sufficient to drive c-Fos expression in

the hippocampus, but with re-presentation of each object, context or animal, less c-Fos is expressed (Bernstein et al., 2019; Bertaina-Anglade et al., 2000; Mahringer et al., 2019). Therefore, in much the same way, administration of $E_2$, DPN, or EGX358 once per day may be insufficiently "novel" to the cells of the regions examined, leading to little cellular activity changes compared to vehicle treatment, and this might expand to the other proteins examined in this study. Importantly, the lack of treatment effects on protein expression and phosphorylation cannot simply be attributed loss of ERβ, as these receptors were similar between all groups in both the DH and mPFC. Although we have previously shown that ERβ levels decline in the hippocampus due to time since OVX, it was found that mice euthanized 2 months following OVX had significantly higher ERβ levels than those euthanized 5 months following OVX (Hanson et al., 2018). Therefore, it is unlikely that significant decline in ERβ would have occurred here, and the current results demonstrate that treatment did not affect ERβ expression.

The effects of stress may also have influenced protein alterations in our regions of interest. Oral gavage is a stressful method of delivering drugs to rodents, resulting in elevated plasma and fecal corticosterone levels (Brown et al., 2000; Vandenberg et al., 2014; Walker et al., 2012). Additionally, repeated behavioral and physiological testing may have contributed to greater stress levels in the mice in this study. In particular, the TST and FST are very aversive and are often used not only as measures of depression-like behaviors, but also as stress-inducing methods (de Kloet & Molendijk, 2016; Krishnan & Nestler, 2011; Pesarico et al., 2020). The current study was designed to reduce impacts of stress on behavioral outcomes by conducting less stressful paradigms (e.g., open field) prior to more stressful paradigms (e.g., forced swim test), and a week separated the FST

and OP tests to reduce accumulated stress. However, it is possible that, by the end of the behavioral testing, mice still had moderate to high levels of stress, although this was not confirmed by corticosterone levels or other markers. Glucocorticoids, such as corticosterone, have profound effects on cellular responses throughout the brain, especially in regions laden with glucocorticoid receptors such as the hippocampus. Both acute and chronic stress can lead to decreases in BDNF expression and phosphorylation of the transcription factor, CREB, which is also a major target of ERK signaling (as reviewed in Duman, 2004; Krishnan & Nestler, 2011). Although estrogenic signaling improves affective outcomes, it is possible that this intersection of signaling affected our molecular results, given that all regions examined are targets of glucocorticoid signaling.

The lack of novelty of treatment and the stress of repeated gavage and numerous behaviors likely culminated in the lack of molecular alterations in the DH, mPFC, amygdala, and hypothalamus. This is further evidenced by the fact that $E_2$ treatment was only modestly anxiolytic in the open field and that $E_2$, DPN, and EGX358 enhanced memory outcomes only relative to chance in both the OP and OR tasks, but not relative to vehicle treatment. However, future studies will be needed to determine whether stress and sensitivity to treatment did indeed affect these results. Namely, future studies utilizing an untreated homecage control group and testing acute treatment styles will be highly informative in parsing the molecular effects of EGX358 treatment.

*Effects on Body Weight and Overall Health*

Finally, we also show here that long-term treatment with $E_2$ or ER$\beta$ agonists does not reduce weight gain following OVX or cause adverse health problems including premature death. Interestingly, none of our estrogenic treatments prevented weight gain over time

58

compared to vehicle. As $E_2$ treatment has previously been shown to reduce OVX-induced weight gain in both mice and rats, it was surprising that $E_2$-treated mice did not exhibit significantly lower body weights than vehicle-treated mice (Couse & Korach, 1999; Torsten Hertrampf et al., 2006; Litwak et al., 2014; Sibonga et al., 1998; Wegorzewska et al., 2008). This lack of effect may be at least partially mediated by our use of a standard diet instead of a high-fat diet, as supported by previous rat and mouse studies (Litwak et al., 2014; Weigt et al., 2012; Yokota-Nakagi et al., 2020). It is not surprising, however, to find that neither DPN nor EGX358 reduced weight gain compared to vehicle, as previous studies report mixed results, such that either no effect of phytoestrogen diets or ER$\beta$ agonist treatment was observed in rats, positive effects were found only under high-fat diet conditions in wild-type compared to ER$\beta$ knockout mice, or negative effects were shown under standard diet conditions in mice (Foryst-Ludwig et al., 2008; T. Hertrampf et al., 2007, 2008; Said et al., 2018; Weigt et al., 2012). These findings suggest a stronger ER$\alpha$-mediated protective role in body weight regulation.

One potential confound with our body weight measurements is that the phytoestrogen content (350-650 mg/kg) of our rodent chow might have obscured effects of long-term estrogen treatments on body mass. Long-term consumption of diets containing phytoestrogens at roughly the same concentrations as our diet alleviate weight gain in OVX rats and mice compared to phytoestrogen-free diets (H. K. Kim et al., 2006; Kishida et al., 2008; Kurrat et al., 2015). However, the only dose to effectively reduce body mass in Kim et al. (2006) was much higher than our standard chow (1500 mg/kg versus 350-650 mg/kg, respectively), suggesting that the dose used here may not affect body mass in mice. It is also possible that the phytoestrogen content in our chow affected

other measures in this study, as previous work has demonstrated that long-term consumption of dietary phytoestrogens at similar or lower concentrations than our chow may confound effects of exogenous $E_2$ on anxiety- and depression-like behaviors and may have anxiolytic and depression-reducing effects similar to $E_2$ treatment in OVX rats (Kageyama et al., 2010; Rodríguez-Landa et al., 2017; Russell et al., 2017). However, that long-term $E_2$ treatment here improved anxiety-like behaviors, and $E_2$, DPN, and EGX358 improved vasomotor and memory outcomes, suggests that OVX mice require higher phytoestrogen concentrations than rats to affect these outcomes, which is supported by Kim et al. (2006). Regardless, a follow-up study examining our administration doses in OVX mice fed phytoestrogen-free diets may be helpful in determining whether diet may have confounded results presented here.

That long-term treatment of EGX358 did not increase body weight or result in premature removal from this study due to health complications or apparent stress suggests little adverse effects on overall health. These data complement our previous findings that a single i.p. injection of EGX358 did not affect liver, heart, or kidney tissues and that EGX358 does not facilitate breast cancer cell proliferation or bind to other nuclear hormone receptors (Hanson et al., 2018). Collectively, these two studies suggest minimal negative effects on general health, although more rigorous testing must be conducted in the future to examine other aspects of overall health (e.g., liver, skeleton, and muscles).

*Conclusions*

In conclusion, long-term oral treatment of young OVX mice with a novel, highly selective ER$\beta$ agonist, EGX358, reduced drug-induced vasodilation and enhanced spatial and object recognition memory without adverse effects on anxiety- or depression-like

behaviors, body weight, or overall health. Surprisingly, no treatment effects were seen in cellular activity markers or synaptic plasticity-related proteins in the DH, mPFC, amygdala, or hypothalamus, regions highly implicated in learning and memory, affective outcomes, and hot flash production. Our behavioral and physiological data expand upon our previous findings in which acute post-training treatment with multiple doses of EGX358 via hippocampal infusion, i.p. injection, or oral gavage enhanced spatial and object recognition memory consolidation in young OVX mice (Hanson et al., 2018). Here, we used the lowest effective oral gavage dose from our previous study (Hanson et al., 2018) and found that EGX358 not only enhances memory but also mitigates drug-induced vasodilation, which expands the potential indications for EGX358 drug development to now include treatment of hot flashes. However, more work is needed to determine the cellular and molecular effects of EGX358 in the brain.

It should be noted that OVX in young rodents does not perfectly recapitulate the human menopausal transition. In particular, OVX causes a very abrupt loss of circulating hormones, including $E_2$, due to the sudden removal of the ovaries, whereas menopause in humans results in a gradual loss of circulating hormones due to ovarian senescence. However, the OVX model is useful preclinically to assess effects of exogenous hormones in the absence of endogenous circulating hormones. Thus, this model is a suitable first step in determining the potential efficacy of EGX358. Future studies will explore the dose-response in more detail, and whether higher doses of EGX358 might reduce preclinical indices of menopause-related anxiety and depression, and test efficacy of EGX358 in middle-aged females. Although aging female rodents do not undergo complete follicular loss or experience drops in gonadotrophin levels (Wise, 2000), they do experience

increases in follicle-stimulating hormone, luteinizing hormone, and $E_2$ prior to a complete cessation of hormonal cycling (Frick et al., 1999; Lefevre & Mcclintock, 1988; Nelson et al., 1995), similar to the menopausal transition in human women. Therefore, the assessment of behavioral and physiological responses to EGX358 in middle-aged female mice will be a valuable next step in the preclinical development of this compound. Additionally, future work will be needed to determine the molecular effects of both acute and long-term treatment of EGX358 in brain regions laden with ERβ, such as the DH and mPFC. Altogether, these results show promise for the development of ERβ-driven HTs, and specifically for EGX358 as a potential therapeutic agent that safely and effectively reduces menopausal symptoms, thereby enhancing the quality of life for older women.

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