Effects of a novel ERβ agonist on proteins related to memory and menopausal symptoms in female mice

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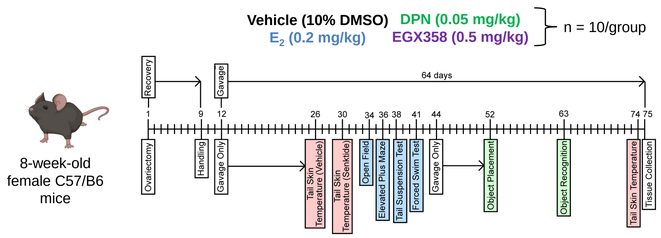
Introduction

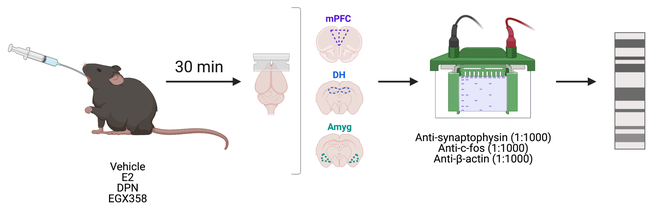
- The menopausal loss of circulating estrogens negatively affects millions of women each year, causing hot flashes, memory decline, anxiety, and depression[1]. Additionally, objective hot flashes are associated with poor verbal memory[2].
- Early treatment with estrogen therapies (ET) does reduce many of the negative symptoms associated with the menopausal transition[3]. However, long-term treatment with ET also promotes health risks, such as the development of cancer[4], effects largely driven by the activation of the alpha (ERα), but not beta (β) estrogen receptor isoform[5]. Thus novel ERβ-selective treatments may prove useful in reducing menopausal symptoms without the adverse risks of ERα activity.
- We have recently developed a novel ERβ agonist, EGX358, which has ~750-fold selectivity for ERβ over ERα and enhances spatial and object recognition memory consolidation when administered acutely to ovariectomized (OVX) mice[6].
- We have also shown that long-term administration of EGX358 via oral gavage reduces drug-induced increases in tail skin vasodilation, a preclinical model of hot flashes, and improves memory in OVX mice[7]. At the completion of this study, tissues were collected from the dorsal hippocampus (DH), medial prefrontal cortex (mPFC), and amygdala 30 min post-final treatment to examine potential protein correlates to physiological and behavioral outcomes.
- **Here, we conducted Western blot analyses of c-fos, a postsynaptic cellular activity marker[8], and synaptophysin, a presynaptic marker of vesicular binding[9], in the DH, mPFC, and amygdala.**
- **We hypothesized that long-term oral treatment with the potent estrogen 17β-estradiol (E2), the commercially available ERβ agonist diarylpropionitrile (DPN), and EGX358 would increase c-fos and synaptophysin expressions in the DH and mPFC, but lower expressions in the amygdala relative to vehicle, reflecting our physiological and behavioral results.**

Methods

Experimental Timeline



Tissue Collection and Western Blots



- Tissue was resuspended in lysis buffer at 50 µg/mL and homogenized
- Homogenates were electrophoresed on 10% Tris-HCl precast gels and transferred to nitrocellulose membranes
- Blocked in 5% milk and incubated with primary antibodies and then with appropriate horseradish peroxidase-conjugated secondary antibody (1:5000)
- Imaged and quantified synaptophysin, c-fos, and β-actin immunoreactivity in ImageLab
- Normalized synaptophysin and c-fos immunoreactivity to β-actin immunoreactivity
- Statistical analyses were conducted using GraphPad Prism 8
- Data were analyzed with one-way ANOVAs to determine between-group differences
- Statistical significance was determined as $p \leq 0.05$

Results

EGX358, E2 and DPN did not alter c-fos or synaptophysin expression in the amygdala

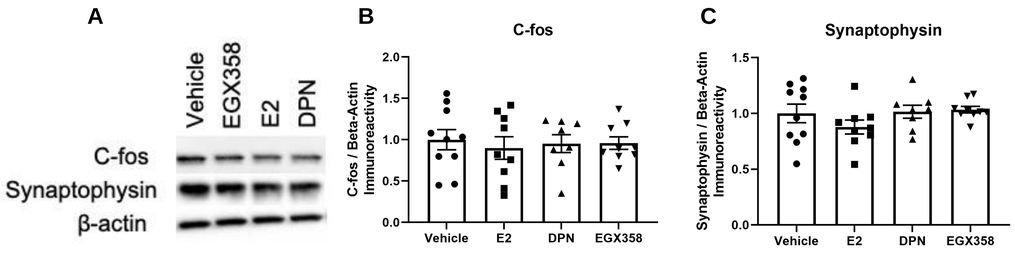


Figure 1. Western blot analysis of c-fos and synaptophysin relative to β-actin in the amygdala. (A) We conducted Western blot analyses of c-fos and synaptophysin in the amygdala following 64 days of treatment with vehicle, E2, DPN, or EGX358, as shown above. We analyzed (B) c-fos and (C) synaptophysin expressions relative to β-actin expression, and all groups were normalized to vehicle treatment; no treatment effects were detected for either protein ($p > 0.05$).

EGX358, E2 and DPN did not alter c-fos or synaptophysin expression in the mPFC

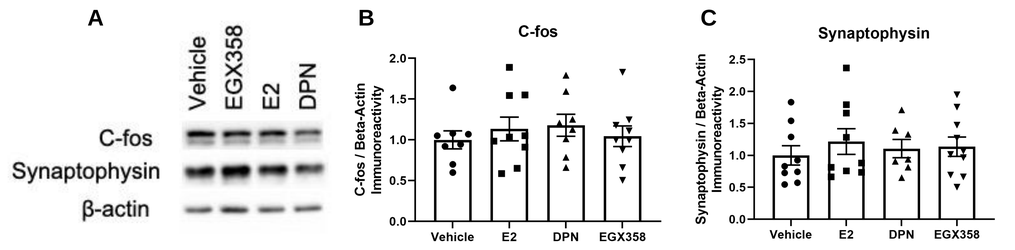


Figure 2. Western blot analysis of c-fos and synaptophysin relative to β-actin in the mPFC. (A) We conducted Western blot analyses of c-fos and synaptophysin in the amygdala following 64 days of treatment with vehicle, E2, DPN, or EGX358, as shown above. We analyzed (B) c-fos and (C) synaptophysin expressions relative to β-actin expression, and all groups were normalized to vehicle treatment; no treatment effects were detected for either protein ($p > 0.05$).

EGX358, E2 and DPN did not alter c-fos or synaptophysin expression in the DH

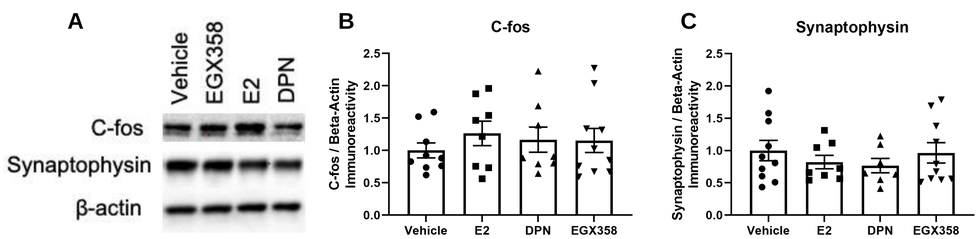


Figure 3. Western blot analysis of c-fos and synaptophysin relative to β-actin in the DH. (A) We conducted Western blot analyses of c-fos and synaptophysin in the amygdala following 64 days of treatment with vehicle, E2, DPN, or EGX358, as shown above. We analyzed (B) c-fos and (C) synaptophysin expressions relative to β-actin expression, and all groups were normalized to vehicle treatment; no treatment effects were detected for either protein ($p > 0.05$).

Summary and Conclusions

Summary

- We expected that long-term treatment with E2, DPN, and EGX358 would increase levels of both c-fos and synaptophysin in the DH and PFC relative to vehicle treatment given that memory was enhanced by these treatments, but lower expression in the amygdala relative to vehicle treatment, in accord with our anxiety-like behavioral results
- Unexpectedly, we found no treatment-induced changes in c-fos or synaptophysin expression in the DH, PFC, or amygdala

Conclusions

- These findings suggest that long-term treatment with E2, DPN, and EGX358 did not have a significantly affect levels of plasticity-related proteins in the amygdala, PFC, or DH
- These results could be due to a number of factors, such as stress of handling and repeated gavage, loss of sensitivity to treatment over time, or wrong time of tissue collection
- Future studies will examine other markers of plasticity, such as glutamate receptor expression, in the DH and PFC, and markers of activity in the hypothalamus to relate to our hot flash-like symptom results.

References

1. Santoro, N., Epperson, N., & Mathews, S. B. (2015). Menopausal symptoms and their management. *Endocrinology and Metabolism Clinics of North America, 44*(3), 497-515. doi:10.1016/j.ecl.2015.05.001
2. Maki, P., Wu, M., Rubin, L., Fornelli, D., Drogos, L., Geller, S., … Conant, R. (2020). Hot flashes are associated with altered brain function during a memory task. *Menopause, 27*(3), 269-277. doi:10.1097/GME.0000000000001467
3. Maki, P. M. (2013). Critical window hypothesis of hormone therapy and cognition: A scientific update on clinical studies. *Menopause, 20*(6), 695-709. doi:10.1097/GME.0b013e3182960cf8
4. Rossouw, J. E., Anderson, G. L., Prentice, R. L., LaCroix, A. Z., Kooperberg, C., Stefanick, M. L., … Ockene, J., Writing Group for the Women's Health Initiative Investigators. (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the Women's Health Initiative randomized controlled trial. *JAMA, 388*(3), 321-333. doi:10.1001/jama.288.3.321
5. Péqueux, C., Raymond-Letron, I., Blacher, S., Boudou, F., Adlanmerini, M., Fouque, M., … Lenfant, F. (2012). Stromal estrogen receptor-a promotes tumor growth by normalizing an increased angiogenesis. *Cancer Research, 72*(12), 3010-3019. doi:10.1158/0008-5472.CAN-11-3768
6. Hanson, A. M., Perera, K. L. I. S., Kim, J., Pandey, R. K., Sweeney, N., Lu, X., … Sem, D. S. (2018). A-C estrogens as potent and selective estrogen receptor-beta agonists (SERBAs) to enhance memory consolidation under low-estrogen conditions. *Journal of Medicinal Chemistry, 61*, 4720-4738. Doi:10.1021/acs.jmedchem.7b01601
7. Fleischer, A. W., Schalk, J. C., Wetzel, E. A., Hanson, A. M., Sem, D. S., Donaldson, W. A., & Frick, K. M. (2021). Long-term oral administration of a novel estrogen receptor beta agonist enhances memory and alleviates drug-induced vasodilation in young ovariectomized mice. *Hormones and Behavior, 130,* 104948. doi:10.1016/j.yhbeh.2021.104948
8. Adams, D., Shen, C., Levenga, J., Basta, T., Eisenberg,S., Mapes, J., Hampton, L., Grounds, K., Hoeffer, C., & Stowell, M. (2017) Synaptophysin is a β-amyloid target that regulates synaptic plasticity and seizure susceptibility in an Alzheimer's Model. *bioRxiv.* doi:10.1101/129551
9. Jaworski, J., Kalita, K., & Knapska, E. (2017) c-Fos and neuronal plasticity: the aftermath of Kaczmarek's theory. *Acta Neurobiologiae Experimentalis 78,* 287–296 doi: 10.21307/ane-2018-027

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