Research Paper

ERβ agonist alters RNA splicing factor expression and has a longer window of antidepressant effectiveness than estradiol after long-term ovariectomy

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Background: Estrogen therapy (ET), an effective treatment for perimenopausal depression, often fails to ameliorate symptoms when initiated late after the onset of menopause. Our previous work has suggested that alternative splicing of RNA might mediate these differential effects of ET. **Methods:** Female Sprague–Dawley rats were treated with estradiol (E2) or vehicle 6 days (early ET) or 180 days (late ET) after ovariectomy (OVX). We investigated the differential expression of RNA splicing factors and tryptophan hydroxylase 2 (TPH2) protein using a customized RT2 Profiler PCR Array, reverse-transcription polymerase chain reaction, immunoprecipitation and behaviour changes in clinically relevant early and late ET. **Results:** Early ET, but not late ET, prolonged swimming time in the forced swim test and reduced anxiety-like behaviours in the elevated plus maze. It reversed OVX-increased (*SFRS7* and *SFRS16*) or OVX-decreased (*ZRSR2* and *CTNNB1*) mRNA levels of splicing factors and ERβ splicing changes in the brains of OVX rats. Early ET, but not late ET, also increased the expression of TPH2 and decreased monoamine oxidase A levels in the dorsal raphe in the brains of OVX rats. In late ET, only diarylpropionitrile (an ERβ-specific agonist) achieved similar results — not E2 (an ERα and ERβ agonist) or propylpyrazoletriol (an ERα-specific agonist). **Limitations:** Our experimental paradigm mimicked early and late ET in the clinical setting, but the contribution of age and OVX might be difficult to distinguish. **Conclusion:** These findings suggest that ERβ alternative splicing and altered responses in the regulatory system for serotonin may mediate the antidepressant efficacy of ET associated with the timing of therapy initiation. It is likely that ERβ-specific ligands would be effective estrogen-based antidepressants late after the onset of menopause.

Introduction

Depression is a leading cause of disease-related disability. The lifetime incidence of major depressive episodes in women is almost twice that of men,[1] and it has been suggested that the higher prevalence of major depressive episodes in women is associated with female-specific reproductive events, such as perimenstrual changes, pregnancy, the postpartum period and menopause.[2] The menopausal transition, for example, appears to represent a period in which some women might be more vulnerable to the development of new-onset or recurrent depressive symptoms and major depressive episodes, resulting in new-onset major depression in approximately 17% of women and minor depression in another 16%.[3] Hormones and neurotransmitters share common pathways and receptor sites in areas of the brain linked to mood, so the fluctuations in sex hormones that mark female reproductive events could influence neurochemical pathways linked to depression.[2] For this reason, the impact of reproductive hormone fluctuations on downstream targets implicated in depression and its treatment is of considerable interest. These include GABAergic and serotonergic neurotransmission, as well as certain neurogenic agents.[4–6]

Estradiol (E2) has been reported to increase the basal firing rate of serotonergic neurons in intact female rats, increase serotonin turnover rate in ovariectomy (OVX) rats, and partially desensitize and decrease the binding ability of the 5-HT1A autoreceptor (a serotonin receptor involved in anxiety and depressive disorders) in intact female rats and OVX monkeys.[7–9] Furthermore, E2 treatment relieved OVX-induced nociceptive hypersensitivity and depression-like behaviours when started 2 days or 4 weeks after OVX.[10,11] The rate-limiting enzyme tryptophan hydroxylase (TPH) for serotonin synthesis and the primary metabolic enzyme monoamine oxidase A (MAO-A) for serotonin degradation are both potential targets for estrogens in the dorsal raphe.

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Submitted Oct. 10, 2017; Revised Feb. 22, 2018; Accepted Mar. 10, 2018; Published online Aug. 14, 2018

DOI: 10.1503/jpn.170199

For example, E2 increased expression of TPH2, the primary isoform of TPH in the brain, specifically in the dorsal raphe of OVX rats.[11,12] In addition, E2 administration is associated with reduced MAO-A expression in the dorsal raphe of OVX macaques,[13,14] and MAO-A levels are significantly increased in the midbrain during the menopausal transition and menopause in humans.[15]

A pivotal issue emerging in the use of estrogen therapy (ET) is the timing of administration. Although beneficial associations between ET and reduced risk of depressive disorders have been reported in both animal and clinical studies,[2,8] some clinical trials in late postmenopausal women have shown no effect of ET on depression and other anxiety symptoms.[16,17] Available studies and our previous work suggest that there may be a critical window of effectiveness in the use of ET for mood improvement: early initiation of ET after cessation of ovarian function sustains the normal protective role of estrogen, but later ET initiation is ineffective or even harmful.[18–20] We know that E2 acts through a number of different estrogen receptors (ERs), including ERα, ERβ, G-protein-coupled ER (also known as GRER or GPR30).[21] The ERβ receptor is widely distributed in the brain, with especially high expression in the dorsal raphe, hippocampus and cortex,[22] and it plays an important role in mediating the antidepressant effect of E2.[23–25] Its main splice variants, ERβ1 and ERβ2, are derived from the same gene transcript via pre-mRNA alternative splicing, a process during the co- or post-transcriptional stage that occurs in more than 90% of multi-exon human genes.[26,27] Our previous work has shown a negative association between E2 effectiveness and the level of the dominant negative ERβ2[28] on increasing cell proliferation and decreasing depression-like behaviour in short- and long-term OVX rats.[20] It suggests that alternative RNA splicing may account for the differential effects of ET shortly after cessation of ovarian function (the time that has been proposed as the starting point of a critical window of effectiveness for ET) and late after cessation of ovarian function (the time after which that window has closed).

The expression and biological function of the splicing factors are significantly changed in the homeostasis of sex hormones.[20,29–32] We know that ERβ is involved primarily in mood and cognitive activities, and the antidepressant effects of E2 are mediated by ERβ.[23–25,33] Our hypothesis is that the use of an ERβ-specific agonist to avoid the activation of ERα, which is involved in several cancer-related adverse effects of ET, will have a longer critical window of antidepressant effectiveness than E2, and will improve quality of life and reduce potential adverse effects in those who receive ET over the long term. In the current study, we attempted to gain evidence to support this hypothesis, exploring TPH2, MAO-A expression, immobility in behavioural tests and the expression of splicing factors as potential molecular mechanisms in response to E2 and ER-specific agonists in female rats after long-term OVX.

Methods

All studies were in compliance with University of Mississippi Medical Center institutional guidelines. Animal-use protocols were approved by the UMMC Institutional Animal Care and Use Committee and conformed with National Institutes of Health guidelines for the use of vertebrate animals.

Animals and groups

Female Sprague–Dawley rats (total $n = 47$; Harlan Laboratories, Inc.) were housed in pairs in a temperature- and humidity-controlled environment. They had free access to food and water and were kept on a 12 h light/dark cycle, with lights on at 6 am and lights off at 6 pm. The rats were randomly divided into 8 subgroups. We used the first 3 groups to assess the effect of early ET, the second 3 to assess the effect of late ET and the last 2 to assess the effect of ER-specific agonists in late ET: sham OVX + vehicle after 6 days ($n = 6$); OVX + vehicle after 6 days ($n = 6$); OVX + E2 after 6 days ($n = 6$); sham OVX + vehicle after 180 days ($n = 9$); OVX + vehicle after 180 days ($n = 5$); OVX + E2 after 180 days ($n = 5$); OVX + DPN after 180 days ($n = 5$); and OVX + PPT after 180 days ($n = 5$).

Bilateral ovariectomy or sham surgery was performed on rats when they were 9 months old, the age at which their estrus cycles are becoming irregular,[34] as previously described.[20] The rats were treated with E2 6 days post-OVX (equivalent to human early postmenopause [early ET]), or E2 or ER-specific agonists 180 days post-OVX (equivalent to 10–20 years postmenopause in humans [late ET]).[35,36] This experimental design prioritized simulating a clinical setting, in which the primary interest was to compare the efficacy of ET close to the onset of menopause or later in menopause.

Treatments

We delivered E2 (30 μg/kg) or vehicle (corn oil) to OVX rats by subcutaneous injection once a day for 2 days, starting on day 7 or day 181 after surgery, to mimic the early or late initiation of ET in humans, respectively. In the last 2 groups[7,8] of OVX rats, we also initiated treatments on day 181, with one group receiving the ERβ-specific agonist diarylpropionitrile (DPN; 100 μg/kg) and the other receiving the ERα-specific agonist propylpyrazoletriol (PPT; 100 μg/kg), both by subcutaneous injection. It has been reported that in behaviour tests measuring depression-like and anxiety-like behaviour, female rats perform best during proestrus, when estrogen levels are highest (about 40 pg/mL).[37] We based the E2 dose used in the current study on our previous findings that 30 μg/kg E2 produced an antidepressant effect in OVX rats receiving early but not late ET.[20] We have also shown that administration of the same dose produced 42 pg/g E2 in brain tissues (wet weight) and 44 pg/mL E2 in serum of OVX rats, similar to E2 levels during proestrus.[38] We chose the doses of DPN and PPT because of their lower transcriptional activity than E2;[39] their effectiveness at these doses has been demonstrated.[40,41]

Statistical analysis

We analyzed the results from the polymerase chain reaction (PCR) array using RT2 Profiler PCR Array data analysis software, version 3.5, on the SABiosciences Web portal. We

assessed the statistical significance of the data from quantitative PCR, Western blot, immunoreactivity in immunohistochemistry, the forced swim test and the elevated plus maze using 1-way analysis of variance and a subsequent Bonferroni post hoc test to examine the effect of ovarian hormone changes in the early or late ET groups. We analyzed the normality of data distribution using a Levene test before the *t* test and analysis of variance. Differences were considered significant at $p < 0.05$.

Results

Estradiol showed no antidepressant effects and no effect on anxiety-related behaviours in female rats when it was initiated 180 days after OVX (late ET), but ERβ-specific agonists did show these effects.

We tested the antidepressant and antianxiety effects of E2 and ER-specific agonists using the forced swim test and the elevated plus maze, respectively, at the time points indicated in Figure 1A. In early ET, OVX significantly decreased swimming time on the forced swim test ($p < 0.01$) and time in open arms (indicating anxiety reduction) in the elevated plus maze ($p < 0.05$) compared with the sham groups; E2 treatment reversed these changes and significantly increased swimming time ($p < 0.05$) and time in open arms ($p < 0.02$) compared with OVX + vehicle (Fig. 1B, a and b). In late ET, we observed no significant difference between sham, OVX, OVX + E2, or OVX + PPT with respect to swimming time or time in open arms in the behavioural tests. Interestingly, OVX + DPN rats showed significantly increased swimming time ($p < 0.05$) and time in open arms ($p < 0.05$) compared to OVX + vehicle rats (Fig. 1B, c and d). Overall, E2 demonstrated a significant effect in early ET ($F_{2,15} = 4.33$, $p < 0.05$ for the forced swim test; $F_{2,15} = 5.347$, $p = 0.01$ for the elevated plus maze). The ERβ-specific agonist DPN demonstrated a significant effect in late ET ($F_{4,30} = 5.98$, $p < 0.01$ for the forced swim test; $F_{4,30} = 3.659$, $p < 0.05$ for the elevated plus maze). Neither E2 nor PPT demonstrated effects in late ET.

Early and late ET differentially regulated splicing factor expression profile

Steroid hormones influence alternative splicing decisions; in turn, products from alternative splicing affect steroid hormone function.[42] We have reported previously that OVX increased a dominant negative splicing isoform, ERβ2.[20] To further understand the splicing factors involved in this process, we investigated the splicing factor expression profiles in OVX rats that received early and late ET. We used a customized RT2 Profiler PCR Array that contained the majority of genes known to regulate alternative splicing so we could analyze rat frontal cortex samples (a gene list in provided in Appendix 1, Table S1, available at jpn.ca/170199-a1).

In the resulting clustergram, most splicing factors from the heterogeneous nuclear ribonucleoprotein family, the arginine/serine-rich (SR) splicing factor family and the RNA binding motif proteins showed an expression pattern that appeared to be differentially regulated by early ET but not by late ET (Appendix 1, Fig. S1). Genes with significant fold changes were further validated using real-time qPCR. These included members of the SR protein family, SR splicing factor 7 *(SFRS7)*, SR splicing factor 16 *(SFRS16)*, zinc finger (*CCCH* type) RNA-binding motif SR 2 *(ZRSR2)* and *CTNNB1*, a gene that regulates cell cycles. We observed only 1 peak in the melt curve analysis from each sample, suggesting unique PCR product and specificity of the primers for *SFRS7, SFRS16, ZRSR2* and *CTNNB1*.

At 6 days post-OVX, expression of *SFRS7* ($p < 0.01$) and *SFRS16* ($p < 0.05$) was significantly increased compared with the sham groups, while expression of *ZRSR2* ($p < 0.05$) and *CTNNB1* ($p < 0.05$) was significantly decreased. When E2 treatment was initiated on day 7 after OVX, it reversed OVX-induced changes by significantly decreasing mRNA expression of *SFRS7* ($p < 0.05$ v. OVX + vehicle) and *SFRS16* ($p < 0.01$ v. OVX + vehicle; $p < 0.05$ v. sham + vehicle), as well as increasing expression of *ZRSR2* ($p < 0.05$ v. OVX + vehicle) and *CTNNB1* ($p < 0.01$ v. OVX + vehicle; $p < 0.01$ v. sham + vehicle; Fig. 2A, C, E and G).

At 180 days post-OVX, expression of *SFRS7* ($p < 0.05$), *ZRSR2* ($p < 0.01$) and *CTNNB1* ($p < 0.05$) was significantly decreased compared with sham; we found no difference in *SFRS16* expression. When E2 treatment was initiated on day 181 after OVX, it had no effect on OVX-induced reduction of *SFRS7* or *CTNNB1* mRNA (Fig. 2B and H), but it dramatically increased mRNA levels of *SFRS16* ($p < 0.01$ v. OVX + vehicle; $p < 0.01$ v. sham + vehicle) and *ZRSR2* ($p < 0.01$ v. OVX + vehicle; $p < 0.05$ v. sham + vehicle; Fig. 2D and F).

To examine the individual roles of ERα and ERβ in the regulation of splicing factors in late ET, we also used DPN and PPT (ERβ- and ERα-specific agonists, respectively; Fig. 2B, D, F, and H). Both DPN and PPT significantly increased *SFRS7* mRNA expression ($p < 0.01$ v. OVX + vehicle for DPN; $p < 0.05$ v. OVX + vehicle for PPT), although it was still significantly lower than the sham group with PPT ($p < 0.05$ v. sham + vehicle); we observed no effect of DPN or PPT on *SFRS16*. For *ZRSR2* and *CTNNB1*, we noted a divergence: DPN dramatically increased mRNA expression of these 2 genes ($p < 0.01$ v. OVX + vehicle; $p < 0.05$ v. OVX + vehicle), and PPT showed no effect. Gene expression values with the different treatments are summarized in Table 1. The statistical significance for *SFRS7* was $F_{2,15} = 6.79$, $p < 0.05$ in early ET and $F_{4,30} = 3.51$, $p < 0.05$ in late ET; for *SFRS16* was $F_{2,15} = 14.61$, $p < 0.01$ in early ET and $F_{4,30} = 5.27$, $p < 0.01$ in late ET; for *ZRSR2* was $F_{2,15} = 5.68$, $p < 0.05$ in early ET and $F_{4,30} = 12.34$, $p < 0.01$ in late ET; and for *CTNNB1* was $F_{2,15} = 16.53$, $p < 0.01$ in early ET and $F_{4,30} = 3.41$, $p < 0.05$ in late ET.

E2 and ER-specific agonists differentially regulated protein expression of 2 main ERβ isoforms in leukocytes of OVX rats

We have demonstrated that ERβ2 expression in circulating leukocytes mirrors the expression profile in brain in OVX rats.[20] To see the isoform expression pattern in circulating leukocytes, an easily obtainable clinical sample from humans, we examined the expression of ERβ and ERβ2 in rat leukocytes of the different treatment cohorts in both early and late

ET (Fig. 3). We found significant treatment effects on ERβ2 expression in both early ET ($F_{2,15} = 4.63$, $p < 0.05$) and late ET ($F_{4,30} = 5.6$, $p < 0.01$). Consistent with our previous findings,[20] OVX significantly increased ERβ2 protein expression both 6 and 180 days after OVX ($p < 0.05$ for both) compared to sham at 6 and 180 days. Early, but not late, E2 administration

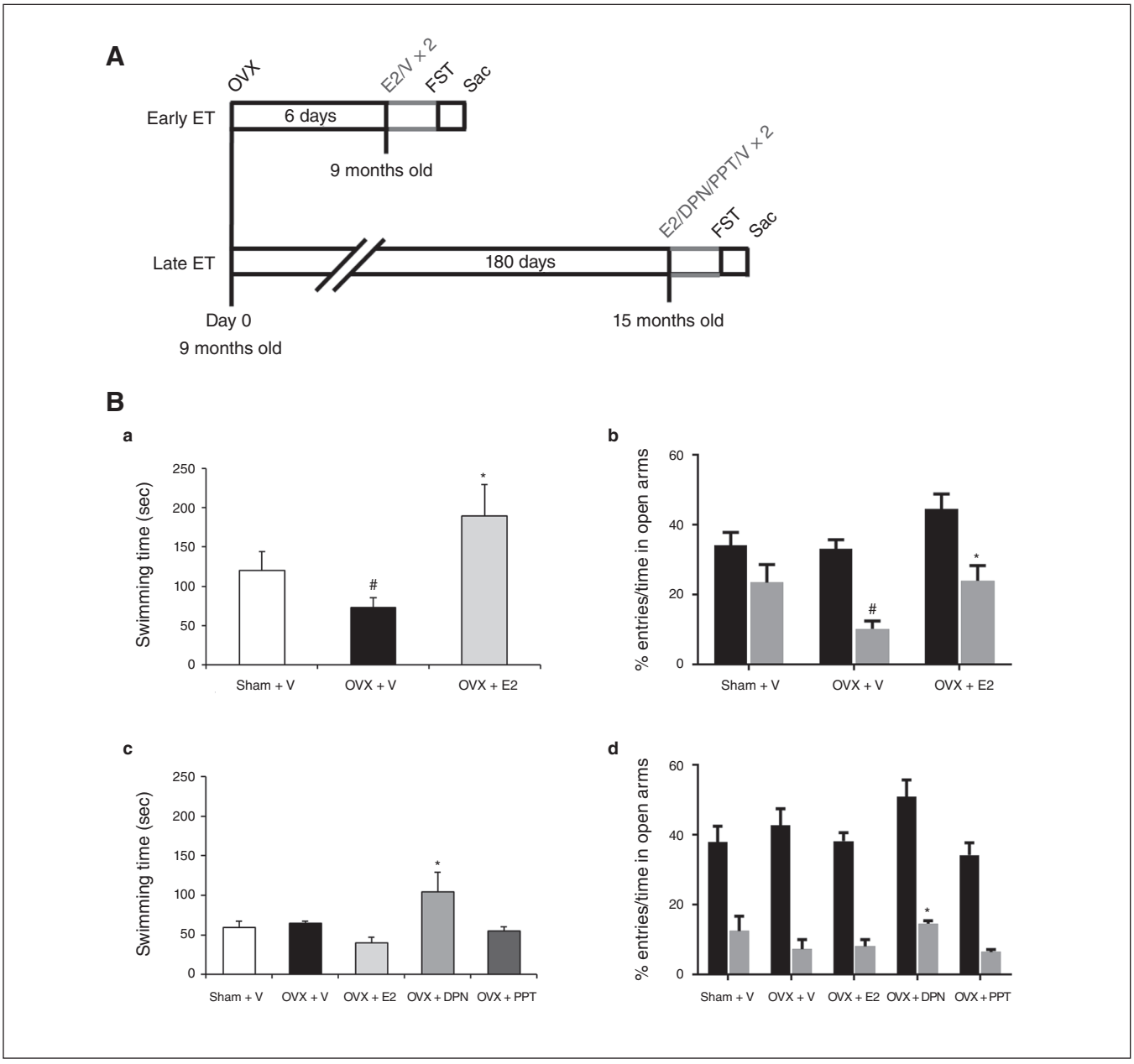

Fig. 1: E2 and ER-specific agonists regulate mobility and anxiety-like behaviours in OVX rats. (**A**) Experimental regimen. Female Sprague–Dawley rats were ovariectomized at day 0 (9 mo of age), when irregular estrous cycles usually begin in laboratory rodents. They were then separated into 2 treatment groups: early and late ET, with different durations of ovarian hormone deprivation (6 d and 180 d). This experimental paradigm mimicked a common clinical setting, in which perimenopausal women (about 50 yr of age) receive ET at different points postmenopause. In the early ET group, OVX rats were treated with either E2 or vehicle (corn oil) at day 7 (equal to 4 mo in humans). In the late ET group, OVX rats were treated with E2, DPN, PPT or vehicle at day 181 (equal to age > 11 yr in humans). After 2 days of treatment, rats were subjected to a forced swim test, and samples were collected on the following day. (**B**) Behavioural tests. The forced swim test (a, c) as an assessment of antidepressant activity and the elevated plus maze (b, d) as an assessment of anxiety-like behaviour were performed in rats treated with vehicle or E2 6 days post-OVX (a, b) or vehicle, E2, DPN or PPT 180 days post-OVX (c, d). Data were analyzed using 1-way ANOVA and a subsequent Bonferroni post hoc test, and are presented as mean ± SEM, $n = 6$ for early ET, $n = 5$ for late ET. #$p < 0.05$ v. sham + V; *$p < 0.05$ v. OVX + V. ANOVA = analysis of variance; DPN = diarylpropionitrile; E2 = estradiol; ER = estrogen receptor; ET = estrogen therapy; FST = forced swim test; OVX = ovariectomy; PPT = propylpyrazoletriol; Sac = sacrifice; SEM = standard error of the mean; V = vehicle.

reversed OVX-induced elevation of ERβ2 expression ($p < 0.05$ v. OVX + vehicle; Fig. 3A and B). Activation of ERβ in late treatment with DPN decreased OVX-induced ERβ2 levels ($p < 0.01$ v. OVX + vehicle; $p < 0.05$ v. sham + vehicle) to levels that were similar to the sham group. In contrast, activation of ERα in late treatment with PPT had no effect on ERβ2 expression (Fig. 3B).

At 6 days post-OVX, ERβ protein expression was similar in the sham + vehicle, OVX + vehicle and OVX + E2 groups. At 180 days post-OVX, we observed a significant treatment

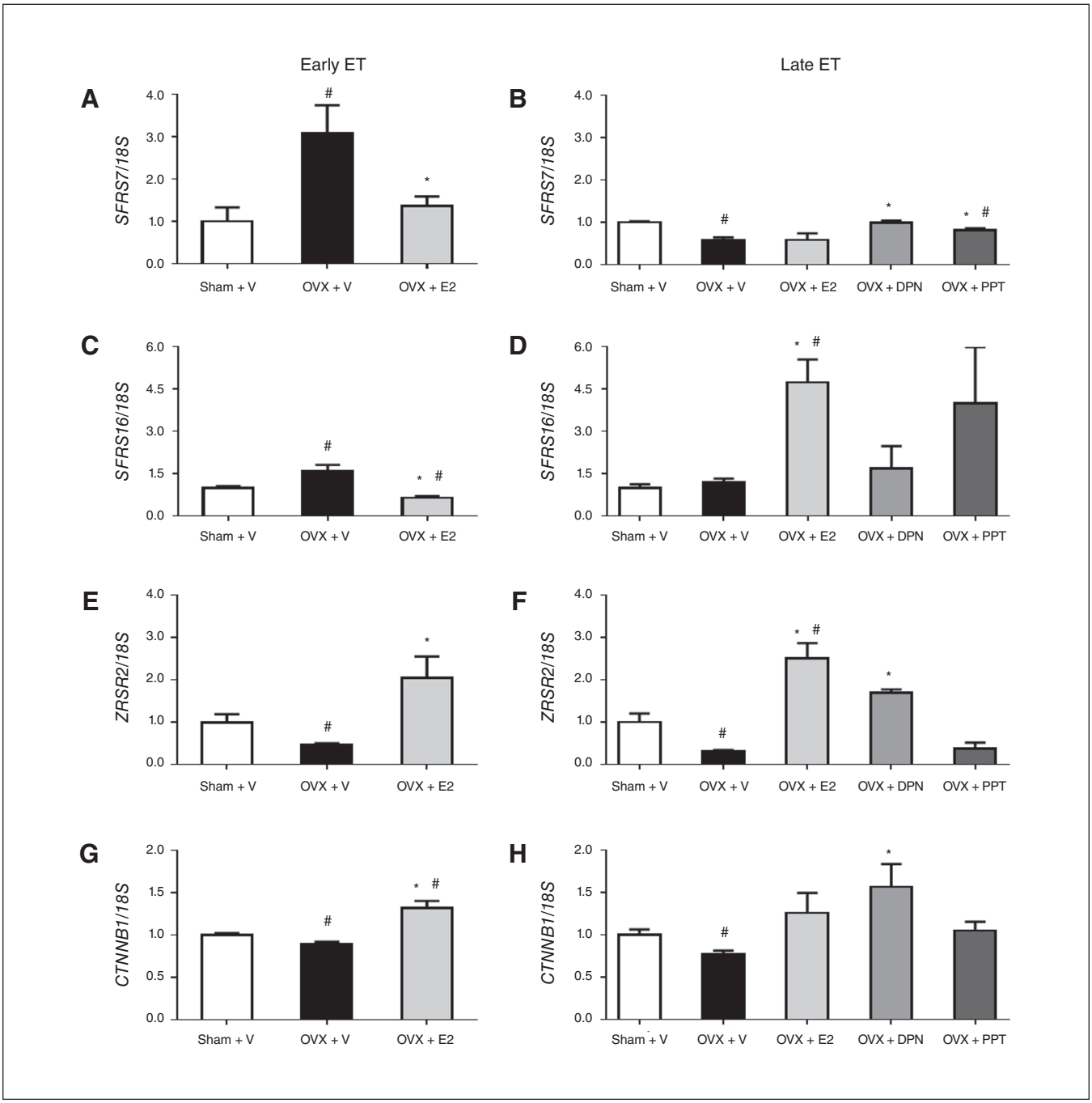

Fig. 2: E2 and ER-specific agonists regulate mRNA expression of splicing factors. We measured *SFRS7, SFRS16, ZRSR2* and *CTNNB1* gene expression in frontal cortex using real-time qPCR with *18S* rRNA expression as an internal control from rats receiving vehicle or E2 6 days post-OVX (**A, C, E, G**) or from rats receiving vehicle, E2, DPN or PPT 180 days post-OVX (**B, D, F, H**). Data were analyzed using 1-way ANOVA and a subsequent Bonferroni post hoc test, and are presented as mean ± SEM. #$p < 0.05$ v. sham + V; *$p < 0.05$ v. OVX + V. ANOVA = analysis of variance; DPN = diarylpropionitrile; E2 = estradiol; ER = estrogen receptor; ET = estrogen therapy; OVX = ovariectomy; PPT = propylpyrazoletriol; qPCR = quantitative polymerase chain reaction; SEM = standard error of the mean; V = vehicle.

Table 1: Average expression of interesting splicing factor genes in OVX rats under different treatment conditions

	Early ET; mean ± SEM (n = 6)		Late ET; mean ± SEM (n = 5)			
Gene	OVX + V	OVX + E2	OVX + V	OVX + E2	OVX + DPN	OVX + PPT
SFRS7	3.09 ± 0.65#	1.37 ± 0.22*	0.58 ± 0.06#	0.58 ± 0.16	0.99 ± 0.05*	0.82 ± 0.03#*
SFRS16	1.61 ± 0.20#	0.64 ± 0.06#*	1.17 ± 0.07	4.94 ± 0.66#*	1.52 ± 0.39	3.82 ± 1.55
ZRSR2	0.46 ± 0.05#	2.05 ± 0.5*	0.31 ± 0.03#	2.51 ± 0.36#*	1.69 ± 0.08*	0.38 ± 0.14
CTNNB1	0.89 ± 0.03#	1.32 ± 0.07#*	0.76 ± 0.03#	1.26 ± 0.24	1.57 ± 0.26*	1.05 ± 0.11

ANOVA = analysis of variance; DPN = diarylpropionitrile; E2 = estradiol; ET = estrogen therapy; OVX = ovariectomy; PPT = propylpyrazoletriol; qPCR = quantitative polymerase chain reaction; SEM = standard error of the mean; V = vehicle.
#$p < 0.05$ v. sham + V; *$p < 0.05$ v. OVX + V. Fold changes of the qPCR results were analyzed with 1-way ANOVA and a subsequent Bonferroni post hoc test.

effect on ERβ expression ($F_{4,30} = 6.01$, $p < 0.01$). The ERβ expression in peripheral leukocytes of the sham and OVX groups was similar and significantly lower than that in rats 6 days post-OVX. Late treatment with E2 ($p < 0.01$ v. OVX + vehicle; $p < 0.01$ v. sham + vehicle), DPN ($p < 0.05$ v. OVX + vehicle; $p < 0.01$ v. sham + vehicle) and PPT ($p < 0.05$ v. sham + vehicle) further decreased it significantly (Fig. 3C and D).

By comparing ERβ2 and ERβ protein expression levels, we found a significant treatment effect for the ERβ2/ERβ ratio in early ET ($F_{2,15} = 5.07$, $p < 0.05$) and late ET ($F_{4,30} = 6.14$, $p < 0.01$). We found a persistently and significantly elevated ERβ2/ERβ ratio 6 days and 180 days post-OVX ($p < 0.05$ and $p < 0.01$, respectively). Early, but not late, E2 treatment reversed this elevation ($p < 0.05$ v. OVX + vehicle). Among all late ET groups, only DPN reduced the ERβ2/ERβ ratio ($p < 0.05$ v. OVX + vehicle). In contrast, E2 and PPT failed to reduce the ERβ2/ERβ ratio: it was still significantly higher than the sham group ($p < 0.01$ v. sham + vehicle for E2; $p < 0.01$ v. sham + vehicle for PPT; Fig. 3E and F). These results indicate that ERβ alternative splicing is differentially modulated by OVX and ER ligands in early and late ET and that activation of ERβ successfully reduces ERβ2 expression in late ET, but E2 does not.

E2 and ER-specific agonists regulated TPH2 mRNA and protein expression in the midbrain of OVX rats

Estradiol acts through ERβ to increase TPH2 expression, possibly by interaction with TPH2 promoter.[43] In both early and late ET, we observed significant treatment effects on *TPH2* mRNA expression ($F_{2,15} = 13.63$, $p < 0.01$ in early ET; $F_{4,30} = 15.23$, $p < 0.01$ in late ET; Fig. 4A and B). We found that *TPH2* mRNA levels in the midbrain of OVX rats were significantly decreased compared with the sham group ($p < 0.01$ in 6-day OVX; $p < 0.001$ in 180-day OVX). Early, but not late, E2 treatment increased *TPH2* mRNA levels 2.5-fold (v. sham, $p < 0.001$) and 4.1-fold (v. OVX, $p < 0.001$). However, late DPN treatment increased *TPH2* mRNA levels 3-fold (v. sham, $p < 0.001$) and 6-fold (v. OVX, $p < 0.001$). We observed no effect with late PPT treatment (Fig. 4B).

In immunoprecipitation analysis, we observed a strong band of TPH2 immunoreactivity at 56 kDa, consistent with the TPH2-expressing SH-SY5Y cell lysate positive control[44] (Fig. 4C). Such a band was absent in the negative control, where no antibody was used during immunoprecipitation. We observed treatment effects in both early and late ET ($F_{2,15} = 9.38$, $p < 0.01$ in early ET; $F_{4,30} = 4.87$, $p < 0.05$ in late ET; Fig. 4D and 4E). We found that OVX consistently decreased TPH2 protein expression ($p < 0.01$ in early ET; $p < 0.05$ in late ET); early, but not late, E2 treatment reversed this OVX-induced TPH2 reduction ($p < 0.01$ v. OVX + vehicle). In late treatments, only activation of ERβ by DPN significantly increased TPH2 protein expression ($p < 0.01$ v. OVX + vehicle) and restored it to levels similar to the sham group (Fig. 4D and 4E).

E2 and ER-specific agonists regulated MAO-A protein expression in the dorsal raphe of OVX rats

We next evaluated the expression of MAO-A, an enzyme involved in serotonin metabolism, by quantifying its immunoreactive signal and distribution in GFAP/NeuN expressing cells in rat raphe nuclei (Fig. 5 and Fig. 6). Fluorescent microscopy showed that the MAO-A signals were localized in both neurons (arrows) and astrocytes (arrowheads) in the dorsal raphe (Fig. 5A and 6A), consistent with our previous report on human postmortem brains.[45] In both early and late ET, OVX significantly increased MAO-A and the GFAP immunopositive signal compared with the sham group ($p < 0.001$ and $p < 0.05$ for early ET; $p < 0.001$ and $p < 0.001$ for late ET); we observed no effect on NeuN expression (Fig. 5B and 6B). Estradiol treatment significantly reduced MAO-A and GFAP expression in early ET ($F_{2,3110} = 8.8$, $p < 0.001$ for MAO-A; $F_{2,8082} = 11.71$, $p < 0.001$ for GFAP), and further increased them in late ET ($F_{4,5248} = 33.66$, $p < 0.001$ for MAO-A; $F_{4,190808} = 11.88$, $p < 0.001$ for GFAP) versus the OVX rats treated with vehicle. In contrast, NeuN expression increased in early ET ($p < 0.01$), but decreased in late ET ($F_{4,6953} = 4.95$, $p < 0.001$). These results were consistent with the TPH2 results and suggested that E2 might only rescue the OVX-induced decrease of serotonin in early ET, but not in late ET. Interestingly, although treatment with PPT (an ERα-specific agonist) demonstrated similar effects to E2 in late ET, treatment with DPN (an ERβ-specific agonist) showed the opposite effect in late ET (Fig. 6B).

Discussion

We found that ET with an ERβ-specific agonist increased swimming time in a forced swim test, and time in open arms in an elevated plus maze task in female rats 180 days after OVX, whereas ET with E2 showed no effects. The

2 alternative RNA splicing components *SFRS7* and *SFRS16* were differentially expressed in the presence and absence of E2 between the mid-aged short-term and old-aged long-term OVX groups; 2 other genes, *ZRSR2* and *CTNNB1*, showed similar responses regardless of age or length of OVX. These splicing factors may play a role in regulating the alternative splicing of ERβ that mediates the expression of TPH2 and MAO-A and ameliorates depressive and anxiety symptoms in OVX rats. Our findings suggest that E2 may regulate splicing factor expression in ovarian hormone deficiency in a time-dependent manner, possibly contributing to decreased OVX-induced ERβ2 elevation.[20,29] After long-term ovarian hormone deficiency and aging, E2 treatment did not show effects on splicing factor expression similar to early ET in reducing ERβ2 levels,[20,29] and it was not as efficient as an antidepressant in late ET. We found that ERβ-specific agonists specifically activated ERβ to regulate the expression of components in the serotonin system and play an effective antidepressant and antianxiety role after long-term OVX in female rats, but E2 did not. Nevertheless, the precise underlying molecular mechanisms warrant further study.

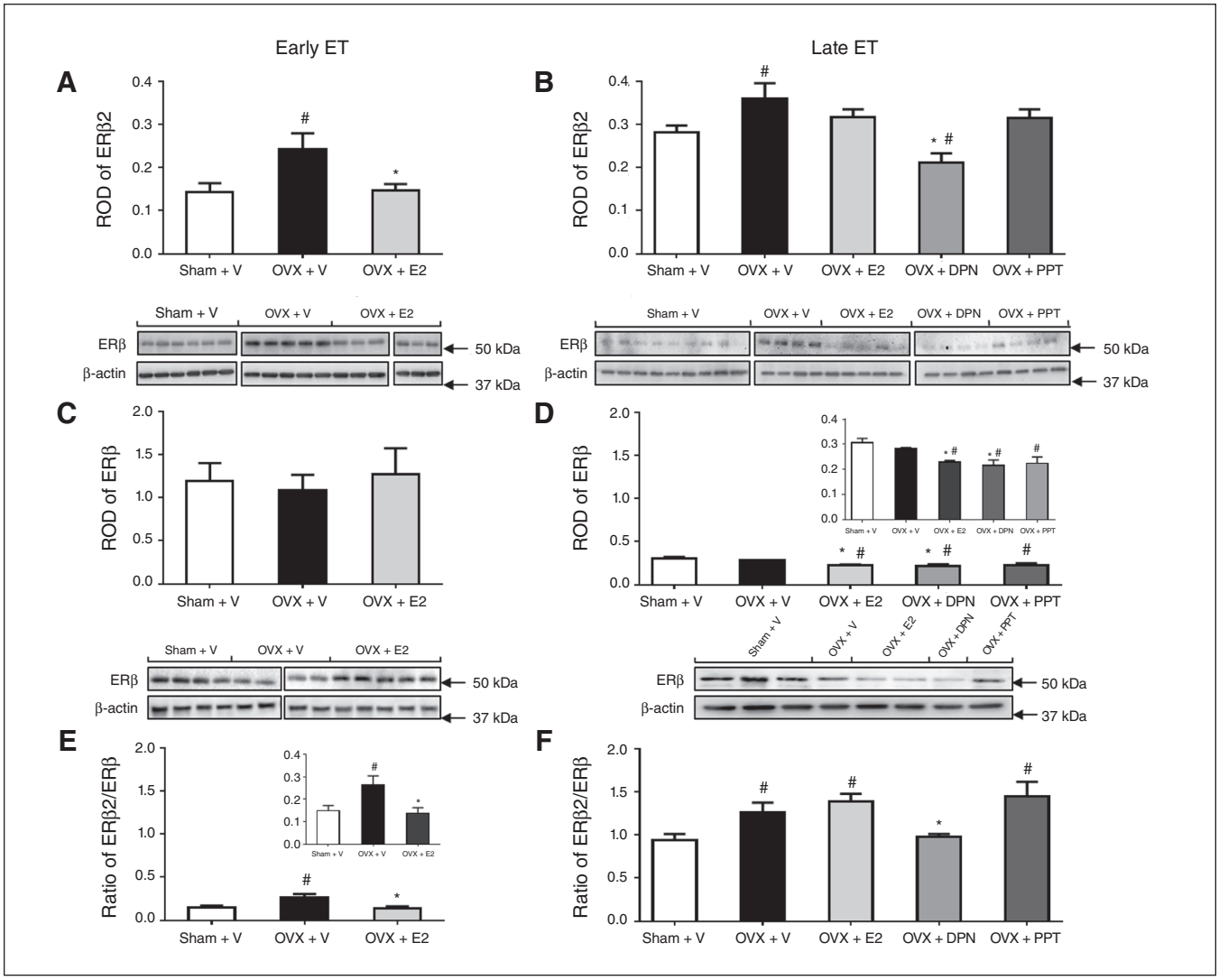

Fig. 3: E2 and ER-specific agonists differentially regulate protein expression of 2 main ERβ isoforms in leukocytes of OVX rats. Leukocytes were extracted from whole blood, and proteins were extracted by RIPA buffer and sonication; total protein was separated by SDS–PAGE. We detected ERβ and ERβ2 protein expression using specific antibodies, with β-actin as the internal control. We compared protein levels of ERβ and ERβ2 in leukocytes from rats receiving vehicle or E2 6 days after OVX (**A, C**) and from rats receiving vehicle, E2, DPN or PPT 180 days after OVX (**B, D**). We compared ERβ2:ERβ protein expression ratio in early ET (**E**) and late ET (**F**) groups. Inserts in **D** and **E** clearly show the differences between each group. Data were analyzed using 1-way ANOVA and a subsequent Bonferroni post hoc test, and are presented as mean ± SEM of ROD; n = 5–6 for early ET, n = 4–9 for late ET. #$p < 0.05$ v. sham + V; *$p < 0.05$ v. OVX + V. ANOVA = analysis of variance; DPN = diarylpropionitrile; E2 = estradiol; ER = estrogen receptor; ET = estrogen therapy; OVX = ovariectomy; PPT = propylpyrazoletriol; RIPA = radioimmunoprecipitation assay; ROD = relative optical density; SDS–PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM = standard error of the mean; V = vehicle.

ERβ alternative splicing regulation by splicing factors

This study showed large fold changes in the RNA profiles of *SFRS7, SFRS16, ZRSR2* and *CTNNB1* in response to OVX and ET. All 4 genes, highly conserved from rodent to human,[46] are involved in many different aspects of general alternative splicing processes and play direct and indirect roles in regulating ERβ alternative splicing. *SFRS7,* a classical SR protein, is constitutively expressed in cells and considered to be an activator of alternative splicing. We found 4 *SFRS7* consensus binding sites (UCAACA) on intron 5, both upstream and downstream of the 54 bp nucleotide retention sequence in ERβ2 pre-mRNA (NCBI database, Gene ID: 25149). *SFRS16,* also called CLK4-associating serine/arginine rich protein (CLASRP), encodes CLK4 protein. It has been reported that CLK4-induced phosphorylation of SR proteins subsequently enhances their splicing ability. *ZRSR2* encodes an essential splicing factor associated with the U2 auxiliary factor heterodimer. It is required for the recognition of a functional 3SS of U2- and U12-type pre-mRNA, and plays a role in network interactions during spliceosome assembly.[47] *CTNNB1* encodes β-catenin protein, which regulates cell proliferation, synaptic plasticity and depression and cognitive function.[48,49] Although β-catenin is not generally considered a splicing factor, it has been reported that it directly caused alternative splicing of ERβ pre-mRNA in colon cancer cells by modulating a

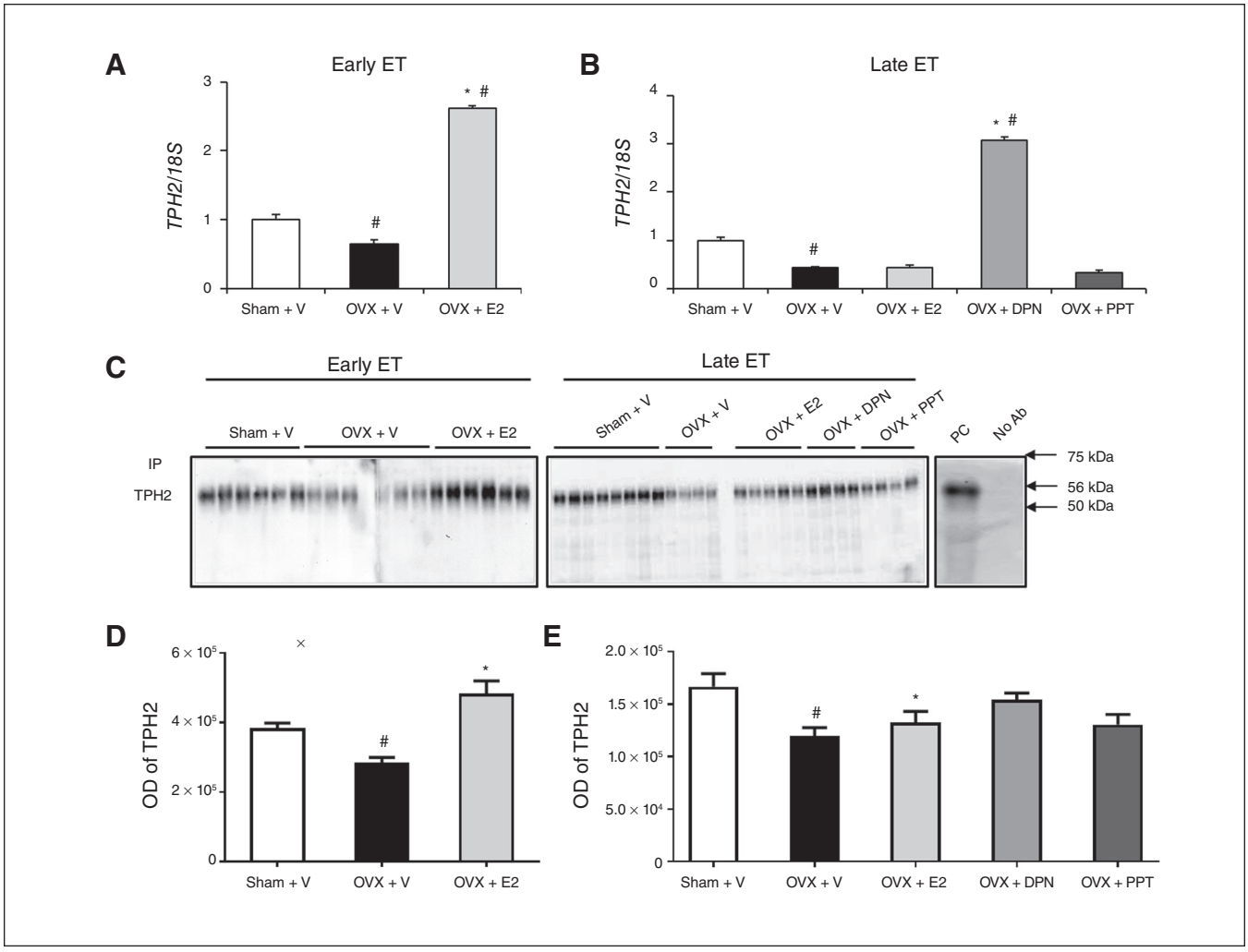

Fig. 4: E2 and ER-specific agonists regulate *TPH2* expression in the dorsal raphe of OVX rats. We measured *TPH2* mRNA expression in rat dorsal raphe using real-time qPCR with *18S* rRNA expression as an internal control from rats receiving ET 6 days post-OVX (**A**) and 180 days post-OVX (**B**). We detected the expression of TPH2 protein in dorsal raphe using SDS–PAGE probed for TPH2 after immunoprecipitation (**C**). We used whole cell lysate from TPH2-expressing SH-SY5Y neuroblastoma cells for positive controls. We used no primary antibody for the negative control in immunoprecipitation. We compared the optical density of TPH2 in rats receiving E2 6 d after OVX (**D**) or rats receiving E2, DPN or PPT 180 d after OVX (**E**). The data were analyzed using 1-way ANOVA and a subsequent Bonferroni post hoc test, and are presented as mean ± SEM; $n = 6$ for early ET, $n = 4$–8 for late ET. #$p < 0.05$ v. sham + V; *$p < 0.05$ v. OVX + V. Ab = antibody; ANOVA = analysis of variance; DPN = diarylpropionitrile; E2 = estradiol; ER = estrogen receptor; ET = estrogen therapy; OD = optical density; OVX = ovariectomy; PC = positive control; PPT = propylpyrazoletriol; qPCR = quantitative polymerase chain reaction; SDS–PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM = standard error of the mean; TPH2 = tryptophan hydroxylase 2; V = vehicle.

high-affinity RNA aptamer.[50] The splicing factors identified in the current study provide potential targets for future investigation to address novel mechanisms of ERβ splicing.

Regulation of TPH2 and MAO-A through ET

In brain, TPH2 is a rate-limiting enzyme for serotonin synthesis, and MAO-A is a major metabolic enzyme for serotonin degradation. Both are potential targets for estrogens to ameliorate depression. Estradiol has been reported to increase expression of TPH2, the primary isoform of TPH in the brain, specifically in the dorsal raphe of OVX rats.[11,12] Furthermore, ERβ agonists specifically display dose-dependent efficacy in vivo in murine dorsal raphe assays for the induction of TPH mRNA associated with antidepressant-like effects;[51,52] in ERβ knockout mice, there was a marked reduction in the expression of TPH in dorsal raphe.[52] During major depressive episodes, MAO-A levels are elevated throughout

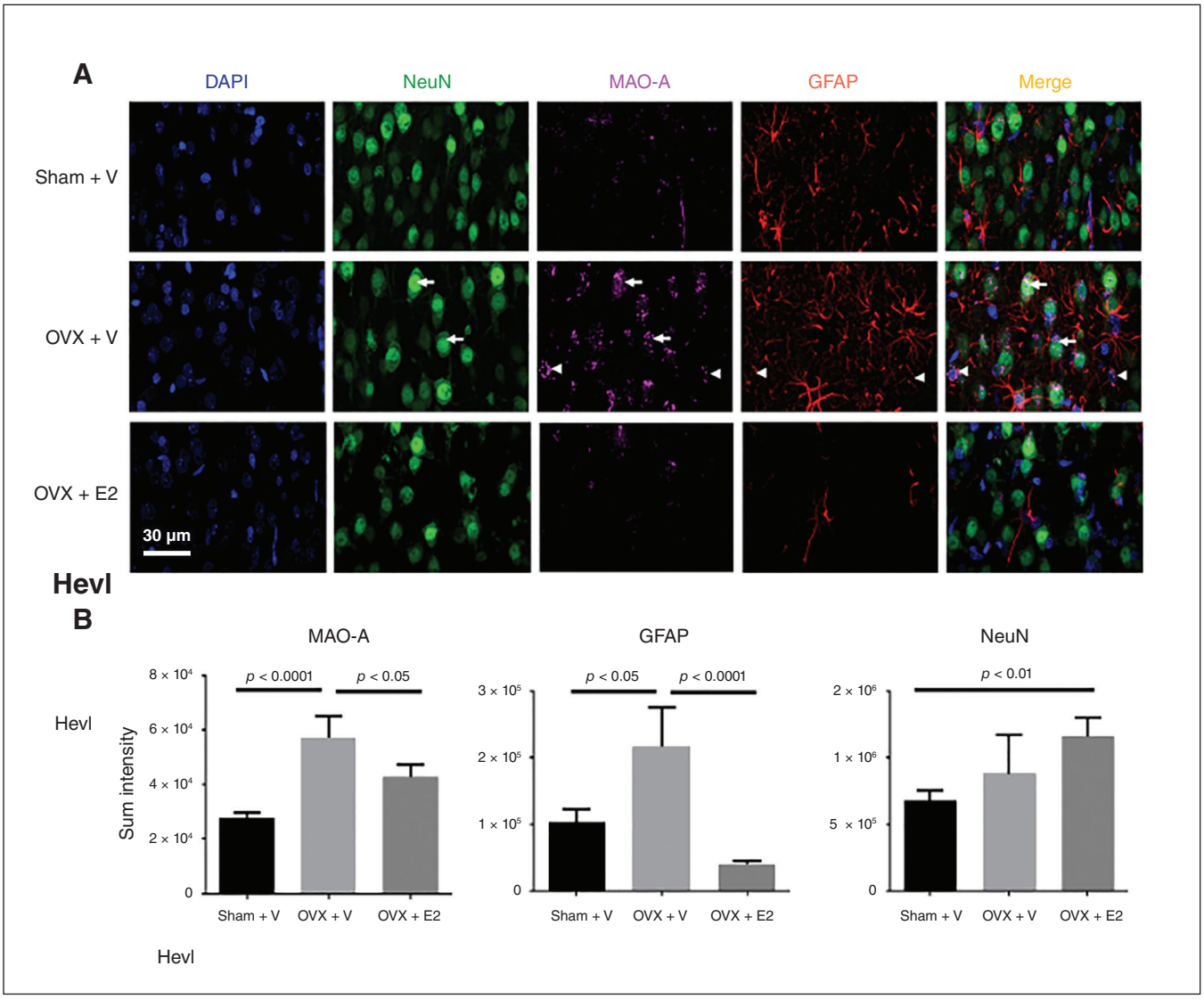

Fig. 5: E2 reduces OVX-induced oxidative stress and glial cell activation in rats (6 d post-OVX). (**A**) Representative immunofluorescence images of dorsal raphe of OVX female rats treated with vehicle, OVX + vehicle and OVX + E2. Nuclei were counterstained with DAPI (blue). Note the nuclear staining of NeuN (green), cytosol punctate staining of MAO-A (magenta) and glial cell–specific staining of GFAP (red). Also note the even distribution of the NeuN staining in the 3 conditions, while both MAO-A and GFAP expressions were increased in OVX rats. Scale bar = 50 μm. Arrows and arrowheads indicate the representative cells expressing MAO-A + NeuN and MAO-A + GFAP, respectively. (**B**) The immunoreactive intensities of MAO-A, GFAP and NeuN in the dorsal raphe of sham + vehicle, OVX + vehicle and OVX + E2 female rats, analyzed using 1-way ANOVA and a subsequent Bonferroni post hoc test. Note the significant increases of MAO-A ($F_{2,3110}$ = 8.80, p < 0.001) and GFAP ($F_{2,8082}$ = 11.71, p < 0.001) expression in the dorsal raphe of OVX rats, but OVX did not change the expression of NeuN ($F_{2,2655}$ = 1.73, p = 0.18); E2 significantly reduced OVX-induced MAO-A and GFAP expression. Although OVX did not change NeuN expression, E2 increased NeuN expression in OVX rats v. sham + vehicle rats. ANOVA = analysis of variance; E2 = estradiol; MAO-A = monoamine oxidase A; OVX = ovariectomy; V = vehicle.

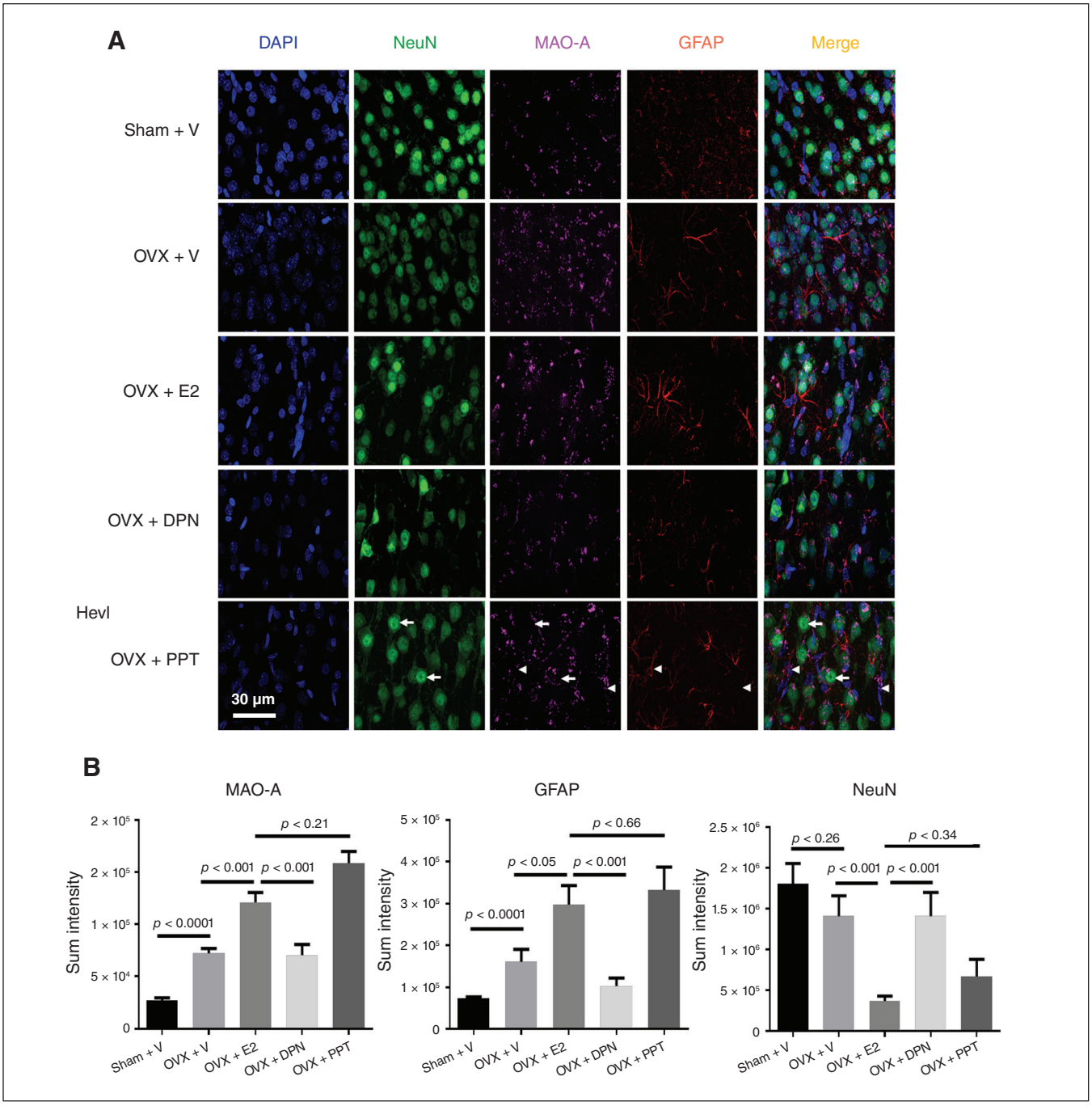

Fig. 6: E2 and ERα-specific agonist increased MAO-A and GFAP expression, and reduced NeuN expression in the dorsal raphe of OVX rats. ERβ-specific agonist ameliorated GFAP expression and maintained NeuN expression in the dorsal raphe of OVX rats (180 d post-OVX). (**A**) Representative immunofluorescence images of the dorsal raphe of OVX female rats treated with vehicle (sham + vehicle), OVX + vehicle, OVX + E2, OVX + DPN and OVX + PPT. Nuclei were counterstained with DAPI (blue). Note the nuclear staining of NeuN (green), cytosol punctate staining of MAO-A (magenta) and glial cell–specific staining of GFAP (red). Scale bar = 50 μm. Arrows and arrowheads indicate the representative cells expressing MAO-A + NeuN and MAO-A + GFAP, respectively. (**B**) We analyzed the immunoreactive intensities of MAO-A, GFAP and NeuN in the dorsal raphe of rats using 1-way ANOVA and a subsequent Bonferroni post hoc test. Note the significant increases of MAO-A ($F_{4,5248}$ = 33.66, p < 0.001) and GFAP ($F_{4,19808}$ = 11.88, p < 0.001) expression in OVX rats; however, OVX did not alter the expression of NeuN ($F_{4,6953}$ = 4.95, p = 0.26). Both E2 and PPT increased MAO-A and GFAP (p < 0.001 and p < 0.05, respectively, for E2; p < 0.001 and p < 0.05, respectively, for PPT) v. OVX, but reduced NeuN expression (p < 0.001 for E2 and p < 0.06 for PPT); DPN ameliorated MAO-A and GFAP expression (p = 0.86 for MAO-A and p = 0.09 for GFAP) and maintained NeuN expression (p = 0.9) in rats 180 d after OVX. ANOVA = analysis of variance; DPN = diarylpropionitrile; E2 = estradiol; MAO-A = monoamine oxidase A; OVX = ovariectomy; PPT = propylpyrazoletriol.

grey-matter regions in the brain, including the midbrain.[45,53] They are similarly elevated in high-risk states for major depressive episodes, including those associated with reduced estrogen levels, such as early postpartum, the menopausal transition and menopause.[15,54] Reducing available MAO-A with antidepressants is effective, but MAO-A inhibitors have many interactions with other medications, so alternative strategies for reducing MAO-A levels need to be therapeutically strategic. Specifically related to this study, E2 administration has also been associated with reduced MAO-A expression in the dorsal raphe of OVX macaques.[13,14] Collectively, these studies suggest that the antidepressant effect of E2 is mediated, at least in part, via specific activation of ERβ to increase TPH2 expression and decrease MAO-A expression in the dorsal raphe.[43]

Manipulations that raise the release of extracellular serotonin are associated with therapeutic response in a number of illnesses, reducing symptomatic behaviours in perimenopause-related major depressive episodes, obsessive–compulsive disorder, anxiety disorders, anger and late luteal phase dysphoric disorder. Therefore, the increase of TPH2 and the reduction of MAO-A in early ET by E2 and late ET by DPN may have therapeutic potential for these conditions. Interestingly, antidepressants, including selective serotonin reuptake inhibitors and MAO-A inhibitors, have all demonstrated the generation of new neurons, as well as the differentiation of newly formed cells (normally with more GFAP) toward neuronal cells (containing NeuN) in hippocampus.[55] The results for the mediation of NeuN and GFAP expression by early E2 and late ERβ agonist treatment in dorsal raphe may suggest a potential neuroprotective role or similar biological potential for other antidepressants in this brain region.

ERβ-mediated therapy in long-term hormone deficiency

Treatment with ERβ-specific agonist DPN has a longer window of antidepressant effectiveness than E2 after long-term OVX. We know that DPN has a 305-fold greater relative binding affinity to ERβ over ERα,[56] and ERβ is highly expressed in brain regions such as raphe nuclei and substantia nigra,[57] while ERα exhibits weak expression in those areas. Functions of ERα and ERβ are greatly overlapped in various tissues, but ERβ is primarily involved in mood and cognitive activity.[23–25,33] In addition to mediating *TPH2* expression, ERβ may underlie other antidepressant effects of E2. Indeed, ERβ agonists, but not ERα agonists, reduced depression-like behaviour in several behavioural tests when administered systemically to OVX rats.[23,25] Such effects were absent in ERβ knockout mice.[24]

The less selective agonists may not be as efficacious as the specific agonists, and their mechanism of action may be more complex. Recent studies have indicated that ethynyl estradiol (an agonist that binds to both ERα and ERβ but has an affinity that is 6 times higher for ERα than ERβ[58]) at doses of 2.5 or 5.0 μg/rat reduced immobility 1 week after, but not 3 weeks after, OVX, even when E2 (1.25 μg/rat) was combined with citalopram (2.5 mg/kg, an antidepressant).[59] However, other studies have reported that in combination with sertraline, E2 reduced immobility in rats 4 or 8 months post-OVX.[60,61] Nevertheless, ERβ-specific agonists produced similar effects to E2 in rats after prolonged loss of ovarian hormones in early ET, potentially reducing the complexity generated by activating ERα, by increasing serotonin synthesis and reducing serotonin metabolism. Indeed, compared with ERβ, ERα is more localized in the reproductive system, such as the ovaries and uterus, so its activation may induce breast and uterine cancer. Although using progesterone replacement along with estrogens can reduce the risk of uterine cancer, progesterone diminishes the antidepressant effects of estrogens, and even exacerbates depressive symptoms.[62] Therefore, ERβ-specific agonists may have a longer critical window of antidepressant effects than E2, with the potential for fewer complications.

Limitations

A limitation of our study is that the contribution of age and OVX were difficult to distinguish in the experimental paradigm. However, we accomplished our goal to investigate differential molecular and behaviour changes in clinically relevant early and late ET, and to identify the ERβ-specific agonist as an effective treatment after long-term ovariectomy.

Conclusion

Our study suggests potential mechanisms associated with the antidepressant efficacy of estrogen. The critical window of effectiveness for ERβ-specific therapy is longer than that for E2 in rats after long-term OVX, and ERβ-specific therapy may be a potential candidate for postmenopausal estrogen therapy with the potential for fewer complications. Further investigation is needed to understand how the splicing factors are modulated in different hormonal environments and how they alter the alternative splicing of target genes, including ERβ.

Acknowledgments: This study was supported by an Alzheimer's Association Investigator Initiated Research Grant (133086), a Carraway foundation grant, a training grant from a center grant from the National Center for Research Resources (P30 GM103328, PI: CS), a component of the National Institutes of Health, and a MIND center subcontract to J.M. Wang. The authors greatly appreciate the comments and suggestions of the members of X. Hou's thesis advisory committee.

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Competing interests: J. Meyer reports grants from Janssen, outside the submitted work. In addition, he has patents on a brain marker and blood markers of MAO-A for predicting mood disorder and on a dietary supplement to prevent postpartum depression and sad mood during high MAO-A states. No other competing interests declared.

Contributors: T. Mosley and J.M. Wang designed the study. X. Hou, S.O. Adeosun, X. Zhao, R. Hill, B. Zheng and R. Reddy acquired the data, which X. Hou, S.O. Adeosun, X. Zhao, R. Hill, B. Zheng, R. Reddy, X. Su, J. Meyer and J.M. Wang analyzed. X. Hou, S.O. Adeosun, X. Zhao, R. Hill and J.M. Wang wrote the article, which all authors reviewed. All authors approved the final version to be published and can certify that no other individuals not listed as authors have made substantial contributions to the paper.

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