Effects of Long-Term Treatment with Estradiol and Estrogen Receptor Subtype Agonists on Serotonergic Function in Ovariectomized Rats

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Key Words

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Abstract

Acute estradiol treatment was reported to slow the clearance of serotonin via activation of estrogen receptors (ER) β and/or GPR30 and to block the ability of a selective serotonin reuptake inhibitor (SSRI) to slow serotonin clearance via activation of ER α . In this study, the behavioral consequences of longer-term treatments with estradiol or ER subtype-selective agonists and/or an SSRI were examined in the forced swim test (FST). Ovariectomized rats were administered the following for 2 weeks: estradiol, ER β agonist (diarylpropionitrile, DPN), GPR30 agonist (G1), ER α agonist (PPT), and/or the SSRI sertraline. Similar to sertraline, longer-term treatment with estradiol, DPN or G1 induced an antidepressant-like effect. By contrast, PPT did not, even though it blocked the antidepressant-like effect of sertraline. Uterus weights, used as a peripheral measure of estrogenic activity, were increased by estradiol and PPT but not DPN or G1 treatment. A second part of this study investigated, using Western blot analyses in homogenates from hippocampus, whether these behavioral effects are accompanied by changes in the activation of specific signaling pathways and/or TrkB. Estradiol and G1 increased phosphorylation of Akt, ERK and TrkB. These effects were similar to those obtained after treatment with sertraline. Treatment with DPN increased phosphorylation of ERK and TrkB, but it did not alter that of Akt. Treatment with PPT increased phosphorylation of Akt and ERK without altering that of TrkB. In conclusion, activation of at least TrkB and possibly ERK may be involved in the antidepressant-like effect of estradiol, ER β and GPR30 agonists whereas Akt activation may not be necessary.

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Introduction

Many of the effects of estrogen are mediated by estrogen receptors (ERs), primarily by ER α and ER β , which are encoded by different genes [1, 2]. These receptors are regionally distributed throughout the body and brain [3]. Some of the regional differences in ER α versus ER β distribution in brain may confer specific functional effects of estradiol (E₂) acting via these receptor isoforms. ER α is predominant in the ventromedial hypothalamus and is related to the facilitatory actions of E₂ on female rodent sexual responding. ER β is predominant in limbic and/or

stress-responsive regions of brain, such as the hippocampus, and is related to the affective behaviors of E₂ [4]. The effects of E₂ in producing anxiolytic-like or antidepressant (AD)-like behaviors through activation of the ER β subtype have been demonstrated using both ER β knock-down and ER β activation approaches [5–10]. Estrogen can act either by a genomic mechanism, using classical nuclear transcription factor receptors [11] or by a nongenomic, less well-characterized membrane receptor signaling mechanism [12]. The genomic pathway requires at least 30–60 min to be manifest and is associated with changes in protein synthesis. Rapid effects of estrogen occurring within seconds to minutes may be mediated by membrane receptors leading to activation of signaling pathways. E₂ has both rapid as well as slower effects on serotonin transporter (SERT) function, indicating the involvement of nongenomic as well as genomic mechanisms in its effects [10, 13].

Recently, a novel G protein-coupled ER, GPR30 (also known as GPER1), has been identified [14]. GPR30 is a seven-transmembrane-spanning G-protein-coupled receptor that shows specific high affinity binding for E₂ and related estrogens and promotes rapid estrogen signaling in a variety of cell types [15]. The GPR30-specific agonist, G1, can reproduce many effects of estrogen [14]. In addition, acute activation of GPR30 by G1 mimicked the AD-like effects of E₂, similar to that observed with an ER β agonist [10].

Using *in vivo* chronoamperometry in young adult ovariectomized (OVX) rats, acute systemic treatment as well as local administration of E₂ produced changes in SERT function, as shown by a decreased basal clearance of serotonin (5-HT); it also prevented the inhibitory effect of the selective serotonin reuptake inhibitor (SSRI) fluvoxamine on 5-HT clearance [13]. These two effects were mediated by different ER subtypes: the AD-like effect of E₂ was due to ER β and/or GPR30 activation whereas its blockade of the ability of an SSRI to slow 5-HT clearance was mediated by activation of ER α [10].

Two major signaling pathways have been associated with the effects of E₂ in the brain: mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) and phosphatidylinositol-3-kinase (PI3K)/Akt (also known as protein kinase B) pathways. The mechanisms of E₂ signaling in the brain are complex, tissue specific and include independent as well as codependent effects through ER α , ER β and GPR30 as well as interactions between ER and other membrane receptors such as insulin-like growth factor receptor (IGF-IR), metabotropic glutamate receptor (mGluR) and the neu-

rotrophin (BDNF and/or NT3) receptor, TrkB [16–19]. In a recent study, it was shown that the acute effects of E₂ on SERT function are mediated by different as well as common signaling pathways. E₂- or diarylpropionitrile (DPN)-induced slowing of 5-HT clearance mediated by ER β was blocked after inhibition of MAPK/ERK1/2 but not that of PI3K/Akt signaling pathways. E₂'s or PPT's inhibition of the fluvoxamine-induced slowing of 5-HT clearance mediated by ER α was blocked after inhibition of either MAPK/ERK1/2 or PI3K/Akt signaling pathways [19]. In addition, estrogen and BDNF seem to share common targets, effects and mechanisms of action [20]. Further, there is evidence for the neurotrophin TrkB receptor being a mediator of the effects of E₂ in the hippocampus [21]. Consistent with this, the blockade of the TrkB receptor abolished the acute effect of E₂ and DPN on 5-HT clearance but had no effect on E₂'s or PPT's inhibition of the fluvoxamine-induced slowing of 5-HT clearance [19].

The brain's serotonergic system is complex, in part due to the diffuse distribution of its multiple receptor subtypes and the various signal transduction pathways regulated by these receptors [22]. Modulation of 5-HT neurotransmission has long been a primary pharmacological target for the treatment of depression and more recently anxiety disorders. SSRIs enhance synaptic 5-HT action by blocking the reuptake of 5-HT [23]. Global enhancement of 5-HT neurotransmission may activate all subtypes of 5-HT receptors in brain, whereas each 5-HT receptor subtype has different and specific functions in defined brain regions [24, 25]. Similar to the signaling of E₂, two major signaling pathways have been implicated in responses underlying AD effects: the PI3K/Akt/glycogen synthase kinase-3 (GSK3) signaling pathway and the MAPK/ERK signaling pathway [24, 26]. In addition to its interaction with estrogen, BDNF and its TrkB receptor are also involved in the mechanism(s) of action of ADs [27]. Phosphorylation of the TrkB catalytic domain (tyrosine residue Y705) is considered to be the initial step in TrkB receptor activation, which further regulates the phosphorylation and activation of other tyrosines, of which Y515 (the docking site of the Shc adaptor protein) and Y816 have been most extensively studied [28]. AD treatment increased TrkB phosphorylation at Y705 as well as at Y816 but not at Y515 [29–31].

Previous studies primarily examined the acute effects of E₂ or subtype-selective agonists. However, E₂ is often given chronically, particularly for adjunctive treatment of postmenopausal depressed patients [32, 33]. Therefore,

Table 1. Treatment with PPT and/or sertraline

	Groups					
	control	PPT (5 µg)	PPT (50 µg)	sertraline	PPT (5 µg)/ sertraline	PPT (50 µg)/ sertraline
Osmotic minipump 1	vehicle	PPT (5 µg)	PPT (50 µg)	vehicle	PPT (5 µg)	PPT (50 µg)
Osmotic minipump 2	vehicle	vehicle	vehicle	sertraline	sertraline	sertraline

Table 2. Treatment with E₂, ER subtype-selective agonists and/or sertraline

	Groups						
	control	E ₂	DPN	PPT	PPT/sertraline	sertraline	G1
Osmotic minipump 1	vehicle	E ₂ (5 µg)	DPN (10 µg)	PPT (50 µg)	PPT	vehicle	G1 (10 µg)
Osmotic minipump 2	vehicle	vehicle	vehicle	vehicle	sertraline	sertraline	vehicle

this study examined the behavioral consequences of longer-term treatments with E₂ or ER subtype-selective agonists and/or an SSRI in the forced swim test (FST). A second part of this study investigated whether behavioral effects of long-term E₂ are associated with changes in the signaling pathways and/or modulatory receptors (TrkB) in the hippocampus accompanying ER activation and/or SSRI-induced AD-like effects. As stated above, the signaling pathways (MAPK/ERK1/2 and PI3K/Akt) and TrkB receptor, selected in this study, are involved not only in signaling through ERs but also are prominently involved in many effects produced by ADs.

Methods

Animals

Ovariectomized (OVX) rats (Sprague-Dawley; 250–280 g, 4 months old, Harlan, Indianapolis, Ind., USA) were housed on a 12:12-hour light/dark cycle with lights on at 07:00 and with food and water provided ad libitum. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used or stress and discomfort to the animals during the experimental procedure. OVX rats were allowed 2–3 weeks' recovery after surgery before the start of the experiment.

Drug Treatment

For the FST experiments, in experiment 1, OVX rats were administered for 2 weeks the following compounds subcutaneously via implantation of one osmotic minipump containing either E₂

(5 µg/day, Sigma-Aldrich, St. Louis, Mo., USA), the ERβ agonist DPN (5 or 10 µg/day, Bio-Techne, Minneapolis, Minn., USA), the GPR30 agonist G1 (5–100 µg/day, Bio-Techne) or the SSRI sertraline (10 mg/kg/day). For experiment 2, examining the effect of the ERα agonist PPT (Bio-Techne) on the AD-like effect of sertraline, two osmotic minipumps were subcutaneously implanted into rats. One was filled with either PPT or vehicle and the other with sertraline or vehicle as shown in table 1. The control group received vehicle which consisted of 25% EtOH/H₂O. In these experiments, 6–14 rats were used in each group.

For the Western blot analysis experiments, OVX rats were implanted with two osmotic minipumps subcutaneously for 2 weeks. The treatments are as shown in table 2. In these experiments, 6 rats were used in each group.

The dose of E₂ used in this study was shown to produce serum E₂ levels seen in proestrus [34]. The initial doses used for ER agonists selected are based on that same study. However, when no effect was obtained with a low dose, higher doses were also tried to make sure that the lack of effect was not due to a dose that was too low. Control groups received vehicle which consisted of 25% EtOH/H₂O.

The behavioral experiments shown in figures 1 and 2 consisted of 2 cohorts. In the first cohort a lower dose of E₂ and ER agonists was used. In the second cohort a higher dose of the agonists was used when necessary. Each cohort had its own control group (n = 7). The results, with low and high doses were combined in the same figures and the two separate control groups were also to provide a larger number of 14. The dose of sertraline was selected as it was shown not only to downregulate the SERT but also to produce an AD-like effect in the FST when given chronically [35–37].

The efficacy of the treatment paradigms were confirmed by body and uterine weight. It has been shown that ERα mediates the attenuating effect of E₂ on body weight gain as well as that of increased uterine weight after E₂ treatment [38–44]. Changes in

Fig. 1. Effect of long-term treatment with E₂, ER β , GPR30 agonists and sertraline (Sertr) in the FST. OVX rats were treated for 2 weeks with E₂, DPN, G1 or sertraline via subcutaneous osmotic minipumps, as described in the Methods section. Mean counts for immobility, swimming and climbing behaviors were sampled every 5 s of the swim test period. Bars and brackets represent the mean value $\pm$ SD, $n = 6-14$ /group. None of the treatment groups had al-

tered climbing behavior (data not shown). **a** Immobility: one-way ANOVA revealed that there was a significant main effect between groups ($F_{6,48} = 17.11$, $p < 0.001$). **b** Swimming: one-way ANOVA revealed that there was a significant main effect between groups ($F_{6,48} = 14.26$, $p < 0.001$). **a, b** Dunnett's post hoc analysis was carried out; * $p < 0.001$, comparing all groups with the control (Ctrl) group.

Fig. 2. Effect of long-term treatment with an ER α agonist (PPT) alone or combined with an SSRI in the FST. OVX rats were treated for 2 weeks with PPT and/or sertraline (Sertr) via subcutaneous osmotic minipumps, as described in the Methods section. Mean counts for immobility, swimming and climbing behaviors were sampled every 5 s of the swim test period. Bars and brackets represent the mean value $\pm$ SD, $n = 6-14$ /group. None of the treatment groups had altered climbing behavior (data not shown). **a** Immobility: two-way ANOVA showed a significant main effect for sertraline ($F_{1,54} = 33.56$, $p < 0.001$) and for PPT ($F_{3,54} = 4.58$, $p < 0.006$)

as well as a significant interaction between sertraline $\times$ PPT ($F_{3,54} = 10.32$, $p < 0.001$). **b** Swimming: two-way ANOVA showed a significant main effect for sertraline ($F_{1,54} = 44.52$, $p < 0.001$) and for PPT ($F_{3,54} = 6.43$, $p < 0.001$) as well as a significant interaction between sertraline $\times$ PPT ($F_{3,54} = 12.80$, $p < 0.001$). **a, b** Dunnett's post hoc analysis was carried out; * $p < 0.001$, comparing each treatment group with the control group (vehicle/vehicle); # $p < 0.001$, comparing PPT (5 or 50 μ g)/sertraline groups with vehicle/sertraline group.

body weight after different treatment were measured (the weight of rats at the end of the treatment was subtracted from their weight just before the start of treatment). In addition, at the end of a long-term treatment with E₂ or ER agonists, the uteri were collected and weighed immediately after removal.

Forced Swim Test

A modified FST procedure from the one described by Cryan et al. [45] was used. On day 15 of chronic treatment (while hormones and/or SSRIs were still administered), a swim test session of 5 min was carried out with no pretest session. This approach is used in order to avoid the extended delay (2 weeks) between the first (pretest) and the second exposure (test) or the impact of hormone being present during the pretest session if pretest and test are separated by the usual 24 h. In addition, it has been shown that positive results with the drugs could be obtained even if no training session was carried out. Rats were placed individually into a Plexiglas cylinder (21 × 46 cm) filled with 25°C water to a depth of 35 cm for a 5-min swim, and behaviors were recorded by a video camera positioned above the tank. A time sampling technique was employed whereby the predominant behavior in each 5-second bin of the test was analyzed. Climbing was defined as upward-directed movements of the forepaws along the side of the swim chamber. Swimming was defined as active movements (usually horizontal) throughout the swim chamber, which also include crossing into another quadrant. Immobility is assigned when there was no active movement other than that necessary to keep the rat's head above the water. The rater was blind with respect to the experimental conditions being scored.

Western Blot Analysis

To prevent changes in the phosphorylation level of proteins due to stress from the FST experiment, another set of rats was used for these experiments. OVX rats were treated as described above. After completion of drug treatment (14 days), the hippocampus was dissected and homogenized in lysis buffer containing 50 mM Tris-HCl, 1 mM EDTA, 0.35% sodium deoxycholate, 150 mM NaCl, 1% Igepal, phosphatase inhibitors and protease inhibitors. After incubation on ice for 30 min, samples were centrifuged for 15 min at 13,000 g. Protein levels of the collected supernates were determined using the Bradford assay. Each sample was run on SDS-PAGE gel and blotted onto a nitrocellulose membrane. The blot was incubated overnight at 4°C with the following primary antibodies: anti-phospho-Y515 (1:1,000; Abcam, Cambridge, Mass., USA), anti-phospho-Y705 (1:1,000; Abcam), anti-phospho-Y816 (1:4,000; Abcam) and anti-TrkB (1:10,000; Neuromics, Edina, Minn., USA), anti-phospho-Akt (S473, 1:1,000; Cell Signaling Technology, Danvers, Mass., USA), anti-phospho-Akt (T308, 1:1,000; Cell Signaling Technology) and anti-Akt (1:1,000; Cell Signaling Technology), anti-phospho-ERK1/2 (1:1,000; Cell Signaling Technology) and anti-ERK1/2 (1:1,000; Cell Signaling Technology). Equal loading was confirmed using anti-β-actin (1:200,000, Sigma-Aldrich) and used for normalization. After three washes, the membranes are incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:20,000, Sigma-Aldrich) for 1 h at room temperature. Enhanced chemiluminescence detection is used followed by an exposure to X-ray film for enhanced chemiluminescence detection. Relative densities were measured using the NIH imageJ program. Phosphoprotein values are normalized and calculated as a ratio against total protein

Table 3. Uterine weight after 14 days' treatment with vehicle, E₂ or ER agonists

	Uterine weight, mg
Vehicle (n = 14)	103±4.7
E ₂ (5 µg) (n = 7)	418±12.7*
DPN (5 µg) (n = 7)	98±1.6
DPN (10 µg) (n = 6)	104±4.4
G1 (5 µg) (n = 7)	103±5.2
G1 (10 µg) (n = 7)	108±2.2
PPT (5 µg) (n = 7)	350±10.3*
PPT (50 µg) (n = 7)	508±14.9*

Values represent means ± SD. OVX rats were treated for 2 weeks with drugs via osmotic minipumps, as described in the Methods section. At the end of treatment/experiment, the uterine horn was dissected and weighed. One-way ANOVA showed a significant main effect ($F_{7,53} = 3,339.38$, $p < 0.001$). Dunnett's post hoc analysis was carried out; * $p < 0.001$, comparing all groups with the vehicle group.

values. The results are shown graphically as a percentage of the control group.

Statistical Analysis

Data were analyzed using SigmaStats (Systat Software Inc., San Jose, Calif., USA). One-way analysis of variance (ANOVA) followed by Dunnett's analysis was used when comparisons were made with the control group (tables 3, 4, 6; fig. 1). Two-way ANOVA followed by Dunnett's analysis was used to compare (1) the effect of PPT within the vehicle and within the sertraline groups and (2) the effect of sertraline within the vehicle and within the PPT groups (table 5; fig. 2). For all the Western blot analyses, where the data are expressed as percentage of control, the Kruskal-Wallis test followed by Dunnett's post hoc analysis was used to compare all groups with vehicle and the sertraline group with the PPT + sertraline group (fig. 3–6). Only when there was a significant main effect and/or interaction effect in the ANOVA were post hoc analyses carried out. Significance was determined at $p < 0.05$.

Results

Long-Term Effects of Treatment with E₂ and ER Agonists on Uterine Weight

Uterine weights were investigated as a peripheral marker of estrogenic activity. As expected, treatment with E₂ and PPT (5 as well as 50 µg) increased uterine weight significantly as compared to the weights in vehicle-treated rats. By contrast, treatment with either DPN or G1 (each at 5 or 10 µg) did not have any effect (table 3).

Fig. 3. Effect of long-term treatment with E₂, DPN, PPT and/or sertraline (Sertr) on phosphorylation levels of Akt (T308) in the hippocampus. OVX rats were treated for 2 weeks with drugs via osmotic minipumps, as described in the Methods section. Western blot analysis of hippocampal levels of total Akt and that of phospho-Akt (T308) were carried out. Representative Western blotting of phospho-Akt and total Akt is shown in the upper inserted panel. A single band was detected at approximately 60 kDa that corresponds to phospho-Akt or total Akt. Bars represent mean value $\pm$ SD of phospho- to total protein ratio (n = 6). Kruskal-Wallis test showed a significant main effect ($F_{5, 30} = 7.52$, $p < 0.001$). Dunnett's post hoc was carried out; * $p < 0.01$, comparing all groups with the control (Ctr) group.

Fig. 4. Effect of long-term treatment with E₂, DPN, PPT and/or sertraline (Sertr) on phosphorylation levels of ERK2 (Y204) in the hippocampus. OVX rats were treated for 2 weeks with drugs via osmotic minipumps, as described in the Methods section. Western blot analysis of hippocampal levels of total ERK and that of phospho-ERK (Y204) were carried out. Representative Western blotting of phospho-ERK1/2 and total ERK1/2 is shown in the upper inserted panel. Bands of phospho-p42 and phospho-p44, ERK-like immunoreactivity at approximately 42 and 44 kDa, respectively, were detected. Only data from phospho-ERK2 are quantified; pERK1 was not quantified due to poor immunoreactivity. Bars represent mean value $\pm$ SD of phospho- to total protein ratio (n = 6). Kruskal-Wallis test showed a statistically significant difference among the treatment groups ($p = 0.005$). Dunnett's post hoc analysis was carried out; * $p < 0.05$, comparing all groups with the control (Ctr) group.

Fig. 5. Effect of long-term treatment with E₂, DPN, PPT and/or sertraline (Sertr) on phosphorylation levels of TrkB (Y705) in the hippocampus. OVX rats were treated for 2 weeks with drugs via osmotic minipumps, as described in the Methods section. Western blot analysis of hippocampal levels of total TrkB and that of phospho-TrkB (Y705) were carried out. Representative Western blotting of Y705 and total TrkB is shown in the upper inserted panel. A single band was detected at approximately 92 kDa that corresponds to Y705 or total TrkB. Bars represent mean value $\pm$ SD of the phospho- to total protein ratio (n = 6). Kruskal-Wallis test showed a significant main effect ($F_{5,30} = 18.61$, $p < 0.001$). Dunnett's post hoc analysis was carried out; * $p < 0.001$, comparing all groups with the control (Ctr) group; # $p < 0.001$, comparing the sertraline group with the PPT + sertraline group.

Fig. 6. Effect of long-term treatment with E₂, DPN, PPT and/or sertraline (Sertr) on phosphorylation levels of TrkB (Y816) in the hippocampus. OVX rats were treated for 2 weeks with drugs via osmotic minipumps, as described in the Methods section. Western blot analysis of hippocampal levels of total TrkB and that of phospho-TrkB (Y816) were carried out. Representative Western blotting of Y816 and total TrkB is shown in the upper inserted panel. A single band was detected at approximately 92 kDa that corresponds to Y816 or total TrkB. Bars represent mean value $\pm$ SD of phosphoprotein to total protein ratio (n = 6). Kruskal-Wallis test showed a significant main effect ($F_{5,30} = 14.07$, $p < 0.001$). Dunnett's post hoc analysis was carried out; * $p < 0.001$, comparing all groups with the control (Ctr) group; # $p < 0.05$, comparing the sertraline group with the PPT + sertraline group.

Table 4. Change in body weight after 14 days' treatment with vehicle, sertraline, E₂, ER α or GPR30 agonists

	Change in body weight, g
Vehicle (n = 14)	+36 $\pm$ 7.5
Sertraline (n = 7)	+31 $\pm$ 9.2
E ₂ (5 μ g) (n = 7)	-1 $\pm$ 15.0*
DPN (5 μ g) (n = 7)	+29 $\pm$ 6.4
DPN (10 μ g) (n = 6)	+41 $\pm$ 9.6
G1 (5 μ g) (n = 7)	+29 $\pm$ 9.0
G1 (10 μ g) (n = 7)	+38 $\pm$ 4.5

Values represent a mean change in body weight $\pm$ SD after chronic treatment with drugs: the weight of the rat at the end of the treatment was subtracted from the weight of the rat at the beginning of the treatment. One-way ANOVA was carried out. There was a significant main effect between groups ($F_{6,48} = 17.04$, $p < 0.001$). Dunnett's post hoc analysis was carried out; * $p < 0.001$, comparing all groups with the vehicle group.

Table 5. Change in body weight after 14 days' treatment with the ER α agonist PPT and/or sertraline

	Change in body weight, g
Vehicle/vehicle (n = 11)	+45 $\pm$ 6.7
PPT (5 μ g)/vehicle (n = 7)	+37 $\pm$ 5.9
PPT (50 μ g)/vehicle (n = 6)	-1 $\pm$ 4.9*
Vehicle/sertraline (n = 11)	+40 $\pm$ 10.8
PPT (5 μ g)/sertraline (n = 7)	+40 $\pm$ 10.4
PPT (50 μ g)/sertraline (n = 7)	+33 $\pm$ 8.3*

Values represent a mean change in body weight $\pm$ SD after chronic treatment with drugs: the weight of the rat at the end of the treatment was subtracted from the weight of the rat at the beginning of the treatment. Two-way ANOVA was carried out for weight change in the sertraline and PPT groups. There was a significant main effect for sertraline ($F_{1,43} = 18.27$, $p < 0.001$) and for PPT ($F_{2,43} = 42.76$, $p < 0.001$) as well as a significant interaction between SSRI $\times$ PPT ($F_{2,43} = 22.46$, $p < 0.001$). Dunnett's post hoc analysis was carried out; * $p < 0.001$, comparing PPT within the vehicle and within sertraline groups; # $p < 0.001$, comparing sertraline within the vehicle and within the PPT groups.

Long-Term Effects of Treatment with E₂, ER Agonists and/or Sertraline on the Change in Body Weight

The initial body weights of the various groups before treatments were not significantly different (data not shown). Tables 4 and 5 show the changes in body weight produced by the various treatments. After 2 weeks of treatment with vehicle, there was a 36- to 45-gram gain in

body weight. Long-term treatment with an ER β or a GPR30 agonist also resulted in a weight gain that was not statistically different from that obtained after treatment with vehicle. However, treatment with E₂ resulted in a small weight loss (-1 g) that was statistically different from the weight gain obtained after treatment with vehicle (table 4). Long-term treatment with sertraline also induced a similar increase in body weight (31–40 g) as that measured in control rats (tables 4, 5). Long-term treatment with the ER α agonist PPT had a dose-dependent effect on body weight. At a low dose (5 μ g), weight gain was equivalent to that obtained in control rats. However, at a higher dose (50 μ g), PPT treatment decreased the body weight significantly compared to the body weight gain seen in vehicle rats. It is interesting to note that in the presence of sertraline the effect of the higher dose of PPT on body weight was not observed (table 5).

Long-Term Effects of E₂, ER Agonists and/or Sertraline in the FST

The behavioral consequences of longer-term treatment with E₂ or ER subtype-specific agonists and/or sertraline were examined in the FST. As expected, chronic treatment with sertraline induced AD-like behavior in the FST, i.e., decreased immobility and increased swimming behavior as compared to that in control rats. Similarly, AD-like effects were found after chronic treatment with E₂, DPN (5 or 10 μ g) or G1 (at 10 but not at 5 μ g) (fig. 1). Increasing doses of DPN (from 5 to 10 μ g) or G1 (from 10 to 100 μ g) did not produce greater behavioral effects (data not shown for G1 above 10 μ g). None of the treatments altered climbing behavior (data not shown).

Chronic treatment with the ER α agonist PPT at doses up to 100 μ g had no effect on immobility or swimming behaviors compared to those measured in control rats. However, the decreased immobility and increased swimming behaviors induced by chronic treatment with sertraline were blocked by long-term treatment with PPT (at either 50 or 100 μ g). PPT at 5 μ g did not alter the sertraline-induced decreased immobility but significantly lowered the sertraline-induced increased swimming behavior in the FST (fig. 2). Again, none of the treatments altered climbing behavior (data not shown).

Long-Term Effects of E₂, ER Agonists and/or Sertraline on Phosphorylation Levels of TrkB and Signaling Proteins

Activation of signaling proteins was assessed by increases in their phosphorylation status in the hippocampus after long-term (14 days) treatment with E₂, ER ago-

Table 6. Effect of long-term treatment with G1 (10 µg/day, 2 weeks) on the phosphorylation levels of Akt, ERK and TrkB in the hippocampus of OVX rats

Groups	pAkt (T308)	pERK2 (Y204)	pTrkB (Y816)
Control (n = 6)	99.6±13	99.5±9	100.4±22
G1 (n = 6)	134.0±15*	141.2±15*	159.8±17*

Values represent means of a percentage of the control group ± SD. OVX rats were treated for 2 weeks with G1 or vehicle (control group) via osmotic minipumps, as described in the Methods section. Western blot analysis of hippocampal levels of total protein and that of phosphoprotein for Akt, ERK and TrkB (Y816) were carried out. Phosphoprotein values are normalized and calculated as a ratio against total protein values. Total protein levels for Akt, ERK and TrkB were not altered by G1 treatment when compared to values in the vehicle treatment group (data not shown). One-way ANOVA followed by Dunnett's post hoc analysis was carried out; * $p < 0.05$, comparing each group with its control group.

nists and/or sertraline, using Western blot analysis. Total protein levels for Akt, ERK and TrkB were not altered by any of the treatments when compared to values in the vehicle treatment group (fig. 3–6). Akt was examined at two phosphorylation sites threonine (T) 308 and serine (S) 473. Phospho-Akt (T308) was significantly increased after treatment with E_2 , G1, PPT, sertraline and the combination of PPT with sertraline; however, treatment with DPN did not alter the phosphorylation level of Akt as compared to control values (fig. 3; table 6). Similar results were obtained with phospho-Akt at S473 (data not shown). Phospho-ERK2 (at tyrosine (Y) 204) was significantly increased in all treatment groups as compared to the values in the control group (fig. 4; table 6). Western blot analysis of TrkB phosphorylation revealed that phosphorylation levels at tyrosine 705 were increased after long-term treatment with E_2 , DPN and sertraline alone or when the SSRI was combined with PPT. While long-term treatment with PPT alone did not alter phosphorylation at Y705 as compared to that seen in the control group, it is interesting to note that the combination of sertraline and PPT produced an increase in Y705 that was significantly smaller than that obtained after sertraline alone (fig. 5). A similar pattern of results were obtained at Y816 on TrkB as treatment with E_2 , DPN, G1 and sertraline alone or sertraline and PPT increased the phosphorylation levels at tyrosine 816, whereas treatment with PPT alone did not have any effect as compared to that seen in the control group (fig. 6; table 6). Again, the combination

of sertraline and PPT produced an increase in Y816 that was significantly smaller than that obtained after sertraline alone. There were no changes in TrkB phosphorylation at Y515 (data not shown).

Discussion

These results demonstrate that long-term treatment of young adult OVX rats with E_2 -, $ER\beta$ - or GPR30-selective agonists induced an AD-like behavior in the FST, i.e., decreased immobility and increased swimming behavior. This effect is similar to that obtained after chronic treatment with the SSRI sertraline. Long-term treatment with the $ER\alpha$ agonist (PPT) had no effect of its own in the FST; however, it blocked the AD-like effect of sertraline. Furthermore, long-term treatment with E_2 , GPR30 agonist or sertraline increased the phosphorylation levels of signaling proteins such as Akt, ERK and that of the TrkB receptor in the hippocampus. Long-term treatment with ER subtype-selective agonists induced similar changes in the levels of phosphorylation of signaling proteins as those obtained with E_2 except that treatment with DPN did not alter phospho-Akt, whereas PPT did not change TrkB phosphorylation levels.

The lack of a gain in body weight associated with increased uterine weight after long-term treatment with E_2 validates the efficacy of the dose of E_2 used (tables 3, 4). ER subtype-selective agonists had different effects on body and uterine weight (tables 3–5). PPT both increased uterine weight and at a dose of 50 µg maintained body weight at a level similar to E_2 consistent with the idea that $ER\alpha$ plays a predominant role in uterine proliferation and maintenance of body weight [38, 43, 46, 47]. DPN and G1 did not alter uterine weight, which is in agreement with previous reports [39, 48]. Weight gain also increased with these agonists to an extent similar to that seen in the vehicle-treated rats.

The AD-like effects of E_2 mediated via $ER\beta$ have been shown in rodents in several studies, using subtype-selective agonists as well as knockout (KO) animals. In acute studies, administration of E_2 or $ER\beta$ -selective ER modulators (SERMs) systemically or directly into the hippocampus decreased anxiety and depressive behavior whereas administration of $ER\alpha$ -specific SERMs did not [9, 49]. Similarly, the AD-like effect of E_2 or DPN in the FST was shown in mice and this effect was lost in $ER\beta$ -KO mice [7]. Long-term treatment of OVX rats with E_2 or DPN induced an AD-like effect in the FST whereas treatment with PPT had no effect [50, 51]. Consistent

with these data, the results obtained in this study indicate that E₂, DPN as well as G1 have an AD-like effect in the FST after long-term treatment. Most importantly, PPT by itself had no effect in the FST, but it blocked the AD-like effect of sertraline (fig. 1, 2). Even though the lower dose of PPT increased uterine weight, PPT at this dose had no effect on the AD-like effect of sertraline (table 4; fig. 2). It is possible that this low dose was not physiologically effective since it was not associated with a lack of body weight gain as was the case for the higher dose (50 µg).

The long-term treatment data from the FST are consistent with the results obtained previously using chronoamperometry to measure the rate of clearance of 5-HT from the CA₃ region of the hippocampus [10, 13]. In these studies, the AD-like effect of E₂ (i.e., slower 5-HT clearance) was due to ERβ and/or GPR30 activation as their specific agonists mimicked the effect of E₂. By contrast, the blockade of E₂ of the ability of SSRI fluvoxamine to slow 5-HT clearance was mimicked only by an ERα agonist [10].

The regulation of the serotonergic system by E₂ leading to the behavioral responses is mediated by ER subtypes that can be located in 5-HT neurons and/or non-5-HT neurons. In general, it is thought that ERβ but not ERα is present in 5-HT neurons. However, the patterns for the expression of the two ER subtypes in 5-HT neurons in the dorsal raphe nucleus (DRN) are species dependent as well as controversial. In rats, it was shown that 5-HT neurons of the DRN contain mRNA for ERβ but not ERα [52]. Distinct groups of 5-HT-immunoreactive neurons in the DRN were found to show ERα and/or ERβ expression in mice but not in rats [53]. In mice, ERβ, but not ERα, was found located within 5-HT neurons [54, 55]. In nonhuman primates, ERβ, but not ERα, seems to be the predominant ER expressed in raphe 5-HT neurons [56–58]. Although GPR30 immunoreactivity was found throughout the brain, it is however not known whether GPR30 is localized in 5-HT neurons [59, 60]. Local activation of ERβ by either E₂ or DPN in the DRN increased the expression of tryptophan hydroxylase-2 (TPH2), the brain-specific rate-limiting enzyme for 5-HT synthesis, and decreased despair-like behavior in the FST; however, it failed to decrease anxiety-like behavior [61]. That study demonstrated that E₂'s regulation of despair-like behavior may primarily involve ERβ located in the DRN whereas its effect in regulating anxiety-like behavior could involve ER located in other brain areas such as the hypothalamus or the amygdala. Further studies will be necessary to investigate whether the ERs mediating the

effect of E₂ on serotonergic function obtained in the present study are located on 5-HT and/or non-5-HT neurons.

Although we have concentrated our effort on the effect of E₂ in the hippocampus, it is important to note that effects of E₂ occur throughout the brain. In addition to the hippocampus, affective behaviors are also modulated by other limbic structures including the amygdala. This brain region contains a high amount of ERs [62]. The amygdala is well known as a brain region important for modulating fear and anxiety behavior [63, 64] and has also been investigated for its importance for affective behavior through a kindling model [65]. The importance of the amygdala for the sex-specific or hormone-dependent modulation of neurobiological changes underlying anxiety disorders has been demonstrated in several studies. For example, lesions of the central nucleus of the amygdala lead to decreased anxiety-type behaviors and decreased neuroendocrine responses to a spectrum of stressors [66, 67]. Systemic as well as intra-amygdala E₂ administration increased antianxiety behavior in OVX rats [49].

There is an interplay between ERs, certain signaling cascades and the TrkB receptor in the behavioral effects of E₂. Activation of dorsal hippocampal PI3K/Akt and ERK signaling pathways is necessary for E₂ to enhance object recognition memory in young as well as middle-aged female mice [68, 69]. Similarly, the activation of ERα, ERβ or GPR30 results in an induction of ERK-Akt signaling pathways mediating neuroprotection and preservation of cognitive function [70, 71]. Both MAPK and PI3K are required in the neuronal actions of estrogen in facilitating female reproductive behavior as lordosis induced by E₂ in rats was abolished in the presence of specific inhibitors of the two signaling pathways [72]. In addition to the importance of these two signaling pathways in the effects of E₂, the activation of the TrkB receptor plays a critical role in E₂-induced neuroprotection from NMDA toxicity [73]. Furthermore, evidence for the TrkB receptor as a mediator of the effects of E₂ in the hippocampus has been demonstrated [18].

In this study, long-term treatment with E₂ increased phosphorylation levels of Akt, ERK and TrkB in the hippocampus, and these effects were similar to those observed after chronic treatment with sertraline (fig. 3–6). Long-term treatment with a GPR30 agonist produced effects similar to those of E₂ (table 6). Interestingly, long-term treatment with ERα and ERβ agonists produced a different pattern of results such that Akt phosphorylation was not changed after DPN treatment whereas PPT treatment did not alter TrkB phosphorylation (fig. 3, 5, 6).

PPT, unlike DPN, did not display an AD-like effect in the FST (fig. 1, 2). Taken together, one might hypothesize that TrkB activation is necessary for an AD-like effect to occur in response to SSRIs, E₂, and ER β - and GPR30-selective agonists. By contrast, activation of Akt may not be required for the DPN-induced AD-like effect in the FST, and perhaps that of E₂ and G1 as well. Recently published findings from acute studies strengthen this hypothesis. Using selective inhibitors of signaling pathways or those of interacting receptors showed that the acute E₂-induced slowing of 5-HT clearance via activation of ER β required MAPK/ERK1/2 signaling pathways and involved interactions with the TrkB receptor. However, the E₂-induced prevention of the ability of an SSRI to slow 5-HT clearance, via activation of ER α , required MAPK/ERK1/2 and PI3K/Akt signaling pathways but not TrkB receptor activation [19]. Perhaps consistent with this idea is also that long-term treatment with the combination of PPT and sertraline significantly reduced the level of phosphorylation of TrkB as compared to sertraline alone (fig. 5, 6), and it also blocked the AD-like effect of sertraline (fig. 2). However, further studies using inhibitors of signaling pathways or those of interacting receptors may offer a better perspective on the impact of signaling pathways and the TrkB receptor in the behavioral effects observed after long term-treatment with E₂- or ER-selective agonists. Nonetheless, these data provide insight into the intracellular activation of specific signaling proteins that accompany these treatments and that could be involved in the AD-like behavioral responses obtained with E₂.

In addition to its interaction with estrogen, BDNF/TrkB is also involved in the mechanism(s) of action of ADs [27] and perhaps the pathogenesis of depression [74]. Evidence that activation of the TrkB receptor is required for a behavioral response typically induced by ADs has been shown [27]. Numerous studies have also implicated MAPK and PI3K pathways in the etiology and treatment of mood disorders. Pharmacological manipulations

of these signaling pathways also affect behavior in models of depression and AD response [24, 26, 75]. These pathways have become major pharmacological targets for putative neuropsychiatric treatments. Our results strengthen the idea that E₂ activation of ER α , ER β or GPR30 leads to behavioral effects that could be due at least in part to activation of ERK, Akt and/or the TrkB receptor in the hippocampus. Such information could be useful in identifying novel drug targets that would bypass ERs.

In conclusion, E₂ has both an AD-like effect as well as an effect that interferes with the efficacy of SSRIs. Different ERs and differences in ER-coupled signaling pathways produce these two different effects of E₂. Understanding the way in which estrogens affect the SERT function in young adult animals is a first step to defining age-related changes in response to hormones. Whether E₂ has effects in a rodent model of peri- or postmenopausal depression similar to effects seen in younger adult rats is under investigation. And if so, then ER β -selective agonists, or novel drugs that would target their signaling pathways, are likely to be more efficacious in postmenopausal depressed patients than E₂.

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