Journal of Steroid Biochemistry & Molecular Biology 122 (2010) 310–317

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Estrogen receptor (ER) subtype agonists alter monoamine levels in the female rat brain

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ARTICLE INFO

Article history:
Received 11 May 2010
Received in revised form 18 August 2010
Accepted 19 August 2010

Keywords:
Estradiol
Estrogen receptors
Rat
Brain
Monoamines

ABSTRACT

We assessed the effects of subtype-selective ER agonists on monoamine levels in discrete regions of the female rat brain. Ovariectomized (ovx) rats were treated for 4 days with vehicle, 17β-estradiol (E; 0.05 mg/kg), an ERβ agonist (C19; 3 mg/kg) or an ERα agonist (PPT; 3 mg/kg) and samples from brain regions were assessed for monoamines and metabolites. We also assessed effects of ERβ modulation on baseline and fenfluramine-induced release of monoamines in hippocampus using microdialysis. In the first study, E and the ERα agonist increased norepinephrine in cortex and all three ER ligands increased it in the ventral hippocampus. Changes in levels of the noradrenergic metabolite, MHPG and the dopaminergic metabolite, DOPAC were noted in brain areas of ER ligand-treated animals. E also increased levels of 5HIAA in three brain areas. In the microdialysis study, there were no differences among groups in baseline levels of monoamines. However, E and the ERβ agonist increased levels of the dopaminergic metabolite, HVA following fenfluramine. In summary, activation of the two nuclear ERs with selective agonists affects monoamine and metabolite levels in discrete brain areas, a number of which are known to play key roles in cognitive and affective function.

1. Introduction

A large body of evidence shows that the gonadal steroid 17β-estradiol (E) modulates activity of numerous neurotransmitter systems in the mammalian brain, including monoaminergic systems [1]. Administration of E alters levels of norepinephrine (NE), dopamine (DA), and serotonin (5HT) and their metabolites in discrete regions of the rat brain (for examples see [2–5]). Modulation of monoamines by E affects many aspects of mammalian physiology including reproductive and non-reproductive process such as learning, memory and affective behaviors [6–8]. Indeed, E has been shown to improve performance in learning and memory tasks in ovariectomized rats and these changes are correlated with changes in monoamines in some brain areas [5].

Estradiol exerts its effects on monoaminergic systems via multiple regulatory mechanisms. For example, E alters levels of enzymes that synthesize DA, NE and 5HT, as well as those that degrade these neurotransmitters [9–13]. Estradiol also regulates levels of monoamine reuptake transporters and receptors (for examples see [14–18]), and coupling of receptors to intracellular second messenger systems [19,20]. Collectively, these findings support the tenet that E acts via multiple mechanisms to alter monoamine levels within the brain.

Two nuclear forms of the ER, termed ERα and ERβ, have been identified. The two receptors share a high degree of homology in the DNA binding domain. Despite significant differences in the ligand binding domains of the receptors, they have similar affinity for E [21,22]. In addition to containing the ligand binding pocket, the ligand binding domains of the receptors also harbor regions

Abbreviations: ovx, ovariectomized; ER, estrogen receptor; E, 17β-estradiol; C19, 4-bromo-9a-butyl-7-hydroxy-1,2,9,9a-tetrahydro-3H-fluoren-3-one; PPT, 4,4′,4″-(4-propyl-{1*H*}-pyrazole-1,3,5-triyl)*tris*phenol; DPN, diarylpropionitrile; HPLC, high performance liquid chromatography; NE, norepinephrine; DA, dopamine; 5HT, serotonin; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5HIAA, 5-hydroxyindole acetic acid; FC, frontal cortex; ACB, nucleus accumbens; ST, striatum; AMYG, amygdala; VHIPP, ventral hippocampus; SN, substantia nigra; VTA, ventral tegmental area; DR, dorsal raphe nucleus; LC, locus coeruleus; TPH2, tryptophan hydroxylase 2; SERT, serotonin reuptake transporter.

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doi:10.1016/j.jsbmb.2010.08.005

important for receptor dimerization. Furthermore, the two ERs can form homo- and hetero-dimer complexes that may act via different intracellular mechanisms to alter gene transcription [23,24]. The ligand binding domains also contain sequence motifs important for interaction with proteins that comprise the transcriptional machinery needed to drive changes in expression of ER-responsive genes. These inherent differences in the ligand binding domains, coupled with differences in the transactivation domains of the two receptors, may allow the ERs to act in different ways to transduce the effects of E.

Both ERα and ERβ are expressed throughout the rat brain in distinct neuronal populations and they can also be co-expressed by neurons [25–28]. Importantly, both ER subtypes are neuroanatomically positioned to regulate the monoaminergic systems. Both ERs are expressed within brainstem NE cell groups [25,26,29,30] and DA cell groups in the hypothalamus [28,31]. The ERβ appears to predominate in DA neurons of the midbrain [32,33]. Serotonergic neurons of the dorsal raphe nucleus of mice and primates [34,35] express ERβ however, the ER complement of serotonergic neurons may differ with species [36,37].

While it is clear that E significantly influences monoaminergic activity in the rat brain, the ER(s) responsible for transducing these effects remains unclear. Imwalle et al. [38] reported that 5HT and DA levels were altered in a number of brain regions of ERβ knockout mice suggesting that ERβ plays a role in modulating some of the effects of E on monoamine levels. Our goal was to determine the effect of ER subtype-selective activation on monoamine and metabolite levels in the female rat brain. We assessed monoamines in discrete regions of the rat brain focussing on areas containing monoaminergic cell bodies and terminal fields associated with cognitive function and affect. Furthermore, we used microdialysis to assess the effects of an ERβ selective modulator on monoamine and metabolite levels the ventral hippocampus of conscious female rats. We identified neurotransmitter- and region-selective effects of ER ligands on monoamines and their metabolites.

2. Materials and methods

2.1. Animal handling and tissue collection

Adult, female Sprague–Dawley rats (~200 g) were purchased (Taconic Farms, Germantown NY or Charles Rivers, North Franklin, CT) ovariectomized (ovx) and shipped 1 week following surgery. After arrival, animals were allowed to acclimate for 1 week prior to the start of the study. They were housed under a 12L:12D light cycle and fed a phytoestrogen-reduced diet (TD96155, Harlan Teklad, Madison, WI). All animal handling procedures were approved by the Institutional Animal Care and Use Committee at Merck & Co., Inc.

2.2. Tissue monoamines

Animals were weighed, assigned to treatment groups (n = 8/group) and dosed s.c. once daily for 4 days with vehicle (100 μl; sesame oil, Sigma, St. Louis, MO), 17β-estradiol (E, 0.05 mg/kg, Sigma), an ERβ subtype-selective agonist (4-bromo-9a-butyl-7-hydroxy-1,2,9,9a-tetrahydro-3H-fluoren-3-one referred to as C19; 3 mg/kg; [39]) or an ERα subtype-selective agonist (4,4′,4″-(4-propyl-{1*H*}-pyrazole-1,3,5-triyl)*tris*phenol (PPT); 3 mg/kg; Tocris Cookson, Inc., St. Louis, MO, Catalog #1426). The ERβ agonist C19 has a potency of 1.8 nM on human ERβ which is similar to that of E, but C19 is >70-fold selective for the ERβ compared to ERα [39]. The dose of the ERβ agonist was based on unpublished data (Merck Research Labs) on the effect of this compound on immature rat uterine weight. The ERα agonist has >400-fold selectivity for ERα compared to ERβ and no activity on the ERβ in transactivation assays [40]. The doses and duration of E and PPT were based on previously published [41] and unpublished (Lubbers) information on the effects of these ligands on uterine weight and CNS indices.

On Day 4, 4 h after the last dose, body weight was measured and the animals were then euthanized with CO_2. Uteri were collected and weighed. Brain tissue was removed, immediately frozen in dry ice and stored at −80 °C until sectioned serially at 300 μm thickness using a cryostat. Sections were freeze-mounted on microscope slides using a drop of ddH_2O and were stored at −80 °C until samples from discrete regions of interest were collected.

Discrete regions of brain were collected by placing microscope slides with affixed tissue sections on a freezing stage maintained at −12 °C and samples from nine brain regions were obtained using a 500 μm diameter punch. The rat brain atlas of Paxinos and Watson [42] served as a reference. A set number of tissue punches per brain area, indicated by the number in parenthesis, were obtained for each animal: frontal cortex (10), nucleus accumbens (6), striatum (8), amygdala (6) ventral hippocampus (12), substantia nigra (6), ventral tegmental area (6), dorsal raphe (6) and locus coeruleus (4). Samples were placed into 1.4 ml Eppendorf tubes and stored at −80 °C.

2.3. Microdialysis

We examined the effects of E and the ERβ agonist C19 on monoaminergic activity in the ventral hippocampus because of the established role of this area in cognitive and affective function (reviewed in [7,43]). Neurons in the ventral hippocampus express both ERs and this area also receives projections from monoaminergic cell groups known to express the receptors [25,44].

Animals were assigned to treatment groups (n = 5–6/group) and dosed s.c. once daily for 4 days with vehicle (100 μl; sesame oil), E (0.05 mg/kg) or C19 (3 mg/kg) as previously described. Microdialysis probes were inserted on Day 3 of dosing. Briefly, rats were anesthetized with 3% isoflurane (in 1% O_2) and implanted with a unilateral microdialysis probe CMA/11 (CMA/Microdialysis, Acton, MA) with a 3 mm dialysis tip in the ventral hippocampus (anteroposterior, −5.3 mm; mediolateral, −4.6 mm; and dorsoventral, −7.5 mm) [42]. The microdialysis probe was slowly lowered into position and was then fixed to the skull by means of three anchoring screws and application of dental acrylic. Immediately following surgery, rats were placed in their testing arena and allowed to recover for a minimum of 14 h.

In vivo microdialysis was performed as previously described [45]. On Day 4 of treatment, rats were tethered to a CMA awake animal system by means of a plastic collar. Following the post-surgery recovery period, perfusion through the dialysis probe with artificial cerebrospinal fluid (aCSF; 145 mM NaCl; 2.7 mM KCl; 1.0 mM $MgCl_2$ and 1.2 mM $CaCl_2$; pH 7.4) was set at 2.0 μl/min. A 2-h stabilization period was then allowed prior to sample collection. Fractions (60 μl) were collected into 250 μl glass vials via a BAS honeycomb fraction collector maintained at 4 °C (BAS HoneyComb; BAS, West Lafayette, IA). Dialysates were collected at 30 min intervals for 3 h to assess baseline levels of neurotransmitters and metabolites. All animals then received fenfluramine (10 mg/kg in saline, i.p., Sigma–Aldrich, St. Louis, MO) which is known to stimulate release of monoamines [46,47] and dialysates were collected for an additional 3 h.

At the completion of sample collection, rats were euthanized by CO_2 inhalation and the microdialysate probes were perfused with approximately 200 μl of cresyl-violet dye. Brains were removed and a coronal cut was made at the level where the probe penetrated the cortex. The resulting coronal surface was imaged using an HP ScanJet 7400L scanner attached to a Compaq IBM PC. Only those

animals demonstrating dye within the ventral hippocampus were included in the analysis.

2.4. Monoamine analysis

2.4.1. Tissue punches

Samples were assessed for monoamine and metabolite levels using high performance liquid chromatography (HPLC) with electrochemical detection and protein levels were determined as previously described [48]. The following neurochemicals were assessed: DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), NE and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG), and 5HT and its metabolite 5-hydroxyindole acetic acid (5HIAA). All samples from a discrete brain area were analyzed within the same HPLC run. Levels of transmitters and metabolites were calculated by reference to standards and an internal standard using peak integration. Values were standardized based on protein levels and expressed as pg/μg protein. In some cases, the majority of neurochemical values (pg/μg protein) within a brain region from a given animal were found to be two or more times the mean values for the group, usually due to low protein concentrations of the sample. Therefore, all values within that region for that animal were removed from the analysis. This resulted in 6–8 animals/group for all tissue monoamine comparisons.

2.4.2. Dialysates

All dialysate samples were analyzed for neurotransmitter content by HPLC within 24 h of collection. Dialysates were assessed for levels of 5HT and 5HIAA, NE, DOPAC and HVA using HPLC coupled to electrochemical detection as described in detail in Lorrain et al. [45]. Peaks corresponding to NE, DOPAC, 5HIAA, HVA and 5-HT eluted with retention times of approximately 4.3, 5.9, 8.8, 12.6 and 16.6 min, respectively. Levels of DA and MHPG in dialysates were not assessed because DA co-eluted with another peak on the chromatogram and could not be reliably measured and MHPG was not detected.

2.5. Statistical analysis

Changes in body weight among groups were compared using repeated measures ANOVA (GraphPad Prism, San Diego, CA) that assessed the main effects of treatment, time and the treatment × time interaction. A one-way ANOVA was used to determine group differences in uterine weight as well as tissue levels of monoamines within region. Bartlett's test was used to assess differences in variance. When unequal variance among groups was detected, data were transformed using either a reciprocal or natural log transformation. For statistical analysis of microdialysis data, data sets were divided into two different time periods, "baseline" (samples 0–180 min) and "fenfluramine challenge" (samples 210–360 min). Monoamine and metabolite concentrations were then compared using repeated measures ANOVA that assessed the main effects of treatment, time period (baseline vs. fenfluramine challenge) and the treatment × time period interaction. All post hoc comparisons were made using Student–Newman–Keuls test and statistical significance was assigned when the p value ≤ 0.05.

3. Results

3.1. Body and uterine weight

Body and uterine weight were monitored as indices of ERα activation [41]. A repeated measures ANOVA revealed a significant effect of treatment ($p = 0.01$), time ($p < 0.001$) and a treatment × time interaction ($p < 0.001$) for changes in body weight.

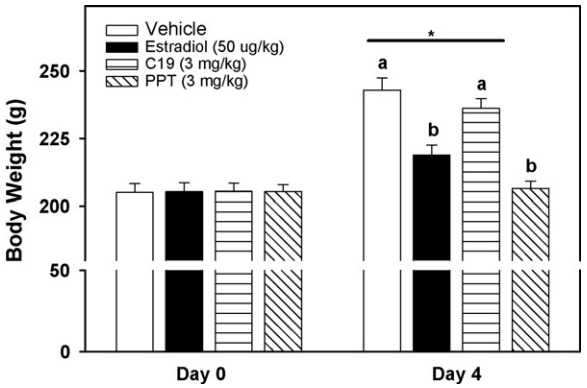

Fig. 1. Effect of ER ligands on body weight over time. Animals ($n = 8$/group) were dosed s.c. for 4 days with vehicle, E (0.05 mg/kg), the ERβ subtype-selective agonist, C19 (3 mg/kg) or the ERα subtype-selective agonist, PPT (3 mg/kg). Mean ± SEM body weight increased over time, except in the group treated with PPT. On Day 4, body weight was greater in animals treated with vehicle or C19 compared to the other two groups. Day 4 body weights of E- and PPT-treated animals were not different from each other. *$p < 0.001$ vs. Day 0; on Day 4, groups with different letters are different from each other, $p < 0.01$.

Body weight increased ($p < 0.001$; Fig. 1) over time in vehicle-, E- and C19-treated animals but not those treated with PPT. On Day 4, body weight was significantly ($p < 0.01$) greater in animals treated with vehicle or C19 compared to E or PPT which did not differ from each other. In contrast to the effects on body weight, uterine weight was increased ($p < 0.001$; Fig. 2) by E and PPT, but not C19 which was not different from vehicle-treated animals.

3.2. Monoamines

3.2.1. Tissue punches

Table 1 shows concentrations of NE and its metabolite, MHPG in the four groups. There was a main effect of treatment on NE concentrations in two areas of the brain. Post hoc analysis showed that both E ($p < 0.05$; 35%) and PPT ($p < 0.01$; 64%) increased levels of NE in the frontal cortex and there was a trend for C19 ($p = 0.07$; 24%) to increase NE in this region as well. All three ER ligands increased NE in the ventral hippocampus ($p < 0.05$; E: 31%; C19: 28%; PPT: 35%). The ERα agonist also increased levels of MHPG in this region ($p < 0.01$; 119%). The ERβ agonist, C19 decreased levels of MHPG in the locus coeruleus ($p < 0.05$; 34%).

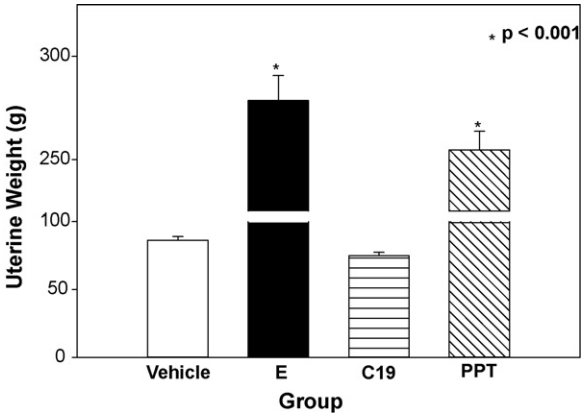

Fig. 2. ER ligand-induced changes in uterine weight. Animals ($n = 8$/group) were dosed s.c. for 4 days with vehicle, E (0.05 mg/kg), the ERβ subtype-selective agonist, C19 (3 mg/kg) or the ERα subtype-selective agonist, PPT (3 mg/kg). By Day 4, treatment with E or PPT, but not C19, increased mean ± SEM uterine weight. *$p < 0.001$ vs. vehicle-treated animals.

Table 1
Effect of ER ligands on the noradrenergic system in discrete regions of the rat CNS.

Region	NE				MHPG			
	Oil	E	C19	PPT	Oil	E	C19	PPT
FC	9.01 ± 1.05	12.18 ± 0.99*	11.16 ± 0.70[b]	14.76 ± 1.70**	20.43 ± 2.26	42.36 ± 11.2	39.10 ± 10.2	33.85 ± 5.07
ACB	0.94 ± 0.19	2.21 ± 0.66	3.39 ± 1.20	0.83 ± 0.22	2.49 ± 0.82	1.65 ± 0.44	1.59 ± 0.38	4.10 ± 2.81
ST	0.14 ± 0.01	0.19 ± 0.04	0.16 ± 0.02	0.24 ± 0.04	0.51 ± 0.08	0.68 ± 0.09	0.59 ± 0.04	0.46 ± 0.09
AMYG	2.24 ± 0.19	2.48 ± 0.14	2.19 ± 0.14	2.55 ± 0.19	3.43 ± 0.68	3.71 ± 0.80	2.57 ± 0.28	2.95 ± 0.27
VHIPP	6.47 ± 0.44	8.45 ± 0.43*	8.25 ± 0.31*	8.71 ± 0.46*	24.07 ± 9.09	13.08 ± 0.64	16.79 ± 3.22	52.67 ± 9.42**,[a]
SN	1.10 ± 0.11	0.86 ± 0.11	0.89 ± 0.05	0.95 ± 0.17	4.49 ± 0.51	2.95 ± 0.31	3.69 ± 0.59	4.06 ± 0.94
VTA	4.23 ± 0.28	5.88 ± 0.73	4.06 ± 0.35	4.66 ± 0.74	7.38 ± 1.35	7.98 ± 0.83	8.49 ± 0.77	7.08 ± 0.65
DR	7.73 ± 0.79	8.64 ± 1.05	7.26 ± 1.01	7.54 ± 0.78	10.39 ± 1.85	9.90 ± 1.47	11.58 ± 2.21	12.44 ± 1.36
LC	31.48 ± 5.42	31.43 ± 4.00	25.26 ± 3.46	35.12 ± 2.86	20.95 ± 1.75	17.89 ± 1.62	13.80 ± 2.14*	20.21 ± 1.53

All data expressed as pg/μg protein and are mean ± SEM, $n = 6–8$ animals/group. All post hoc comparisons are vs. vehicle-treated animals. FC, frontal cortex; ACB, nucleus accumbens; ST, striatum; AMYG, amygdala; VHIPP, ventral hippocampus; SN, substantia nigra; VTA, ventral tegmental area; DR, dorsal raphe nucleus; and LC, locus coeruleus.
[a] Reciprocal transformed.
[b] $p = 0.07$.
* $p < 0.05$
** $p < 0.01$.

Table 2
Effect of ER ligands on the serotonergic system in discrete regions of the rat CNS.

Region	5HT				5HIAA			
	Oil	E	C19	PPT	Oil	E	C19	PPT
FC	10.38 ± 1.90	11.71 ± 2.46	13.61 ± 2.16	12.09 ± 1.57	3.79 ± 0.88	8.96 ± 2.20	4.72 ± 0.84	12.71 ± 6.79
ACB	4.10 ± 0.87	3.97 ± 0.57	3.40 ± 0.45	3.53 ± 0.68	1.08 ± 0.11	1.29 ± 0.12	1.15 ± 0.06	1.11 ± 0.07
ST	0.36 ± 0.05	0.51 ± 0.09	0.42 ± 0.06	0.49 ± 0.05	1.90 ± 0.12	2.58 ± 0.21*,[a]	2.06 ± 0.10	2.68 ± 0.45
AMYG	2.71 ± 0.43	3.11 ± 0.20	2.21 ± 0.27	2.79 ± 0.11	2.62 ± 0.35	2.45 ± 0.32	2.05 ± 0.15	2.36 ± 0.18
VHIPP	5.85 ± 0.51	6.28 ± 0.32	6.38 ± 0.34	6.74 ± 0.82	3.35 ± 0.09	4.61 ± 0.40*	4.07 ± 0.13	3.88 ± 0.39
SN	7.19 ± 0.67	6.43 ± 0.58	6.68 ± 0.63	7.18 ± 0.67	4.39 ± 0.81	4.19 ± 0.28	3.94 ± 0.54	3.70 ± 0.28
VTA	5.62 ± 0.20	7.76 ± 1.04	5.75 ± 0.24	6.53 ± 0.51	7.65 ± 0.45	10.61 ± 0.47**	7.89 ± 0.34	9.14 ± 0.55
DR	11.22 ± 0.90	14.36 ± 1.61	11.91 ± 1.29	13.21 ± 1.01	7.46 ± 0.53	9.85 ± 1.14	9.42 ± 1.66	9.09 ± 0.85
LC	4.88 ± 0.52	5.03 ± 0.42	5.18 ± 0.32	4.73 ± 0.20	5.86 ± 0.46	6.34 ± 0.18	6.75 ± 0.54	6.49 ± 0.64

All data expressed as pg/μg protein and are mean ± SEM, $n = 6–8$ animals/group. All post hoc comparisons are vs. vehicle-treated animals. FC, frontal cortex; ACB, nucleus accumbens; ST, striatum; AMYG, amygdala; VHIPP, ventral hippocampus; SN, substantia nigra; VTA, ventral tegmental area; DR, dorsal raphe nucleus; and LC, locus coeruleus.
[a] Reciprocal transformed.
* $p < 0.05$.
** $p < 0.01$.

Table 2 describes ER ligand-induced changes in serotonergic parameters. There were no significant effects of ER ligands on 5HT levels. In contrast, there was a main effect of treatment on 5HIAA in a number of regions. Specifically, E increased 5HIAA in the striatum ($p < 0.05$; 36%), ventral hippocampus ($p < 0.05$; 38%) and ventral tegmental area ($p < 0.01$; 39%). There were no statistically significant effects of the ER subtype-selective agonists on 5HT or 5HIAA in the areas examined.

There were no statistically significant effects of the ER ligands on levels of DA or the DA metabolite HVA (Table 3). There was a main effect of treatment on levels of the DA metabolite DOPAC in frontal cortex and amygdala. Post hoc comparison of treatment effects in these two areas showed that PPT increased levels of DOPAC in cortex ($p < 0.05$; 72%) whereas C19 decreased it in the amygdala ($p < 0.05$; 42%).

3.2.2. Dialysates

There was a significant main effect of fenfluramine challenge (time period; $p < 0.001$) for a number of monoamines or their metabolites but no overall effect of treatment for any of the analytes. Baseline levels of the monoamines and metabolites were not different among vehicle-, E- or C19-treated groups. Fenfluramine challenge significantly ($p < 0.001$) increased mean levels of 5HT, NE and HVA relative to baseline (Fig. 3). The increase in 5HT concentrations following fenfluramine challenge did not differ among treatment groups. While levels of NE in C19- and E-treated groups appeared to be somewhat greater following fenfluramine challenge compared to vehicle controls, these differences were not statistically significant. For HVA, there was also a significant treatment × time interaction (Fig. 3C; $p < 0.05$); HVA levels were significantly ($p < 0.05$) increased in E- and C19-treated animals following fenfluramine challenge but not in vehicle-treated animals relative to baseline. Fenfluramine did not induce statistically significant changes in DOPAC or 5HIAA in any of the groups (data not shown).

4. Discussion

The aim of this study was to determine the effects of selective activation of the two nuclear ERs on monoaminergic neurotransmitter and metabolite levels in the rat brain. ER isoform-selective changes in monoamines and their metabolites were assessed in discrete regions throughout the female rat brain as well as via microdialysis in the ventral hippocampus. Activation of the two ER isoforms increased levels of NE and also affected metabolism of NE and DA in select brain regions. In contrast, E alone affected levels of the serotonin metabolite 5HIAA. These findings support the idea that both ERα and ERβ mediate the effects of E on catecholaminergic systems in the female rat brain.

All three ER ligands increased tissue content of NE in the ventral hippocampus. Our previous work demonstrated that long-term treatment with E increases NE in the CA3 region of the hippocampus [49]. We have evidence to suggest that the commercially available ERβ agonist diarylpropionitrile (DPN) also affects noradrenergic parameters in different regions of the hippocampus [50]. The present findings extend these observations by demonstrating

Table 3
Effect of ER ligands on the dopaminergic system in discrete regions of the rat CNS.

Region	DA				DOPAC			HVA			
	Oil	E	C19	PPT	Oil	E	C19	PPT	Oil	E	C19	PPT
FC	6.36 ± 1.85	6.90 ± 2.42	6.81 ± 1.41	6.13 ± 2.53	66.02 ± 10.6	77.25 ± 7.08	66.48 ± 4.4	113.38 ± 17.9[*,a]	9.47 ± 3.17	19.83 ± 9.34	5.49 ± 0.53	6.74 ± 1.45
ACB	51.34 ± 2.41	53.80 ± 3.20	48.82 ± 4.35	51.10 ± 2.79	22.04 ± 5.18	15.00 ± 0.97	16.55 ± 2.6	15.67 ± 0.92	6.13 ± 0.42	5.56 ± 0.42	5.40 ± 0.64	6.21 ± 1.05
ST	38.15 ± 2.99	39.64 ± 1.22	36.50 ± 1.75	37.82 ± 1.49	8.65 ± 0.52	10.53 ± 0.8	8.38 ± 0.39	9.30 ± 0.60	5.25 ± 0.39	6.02 ± 0.54	5.40 ± 0.44	4.78 ± 0.35
AMYG	0.98 ± 0.15	0.73 ± 0.08	0.68 ± 0.11	0.81 ± 0.10	0.56 ± 0.07	0.41 ± 0.05	0.32 ± 0.05[*]	0.39 ± 0.05	1.01 ± 0.23	0.61 ± 0.05	1.19 ± 0.41	0.74 ± 0.13
VHIPP	1.08 ± 0.38	1.40 ± 0.15	1.60 ± 0.21	1.63 ± 0.38	0.31 ± 0.09	0.19 ± 0.01	0.39 ± 0.04	0.46 ± 0.12	0.43 ± 0.07	0.53 ± 0.07	0.47 ± 0.03	0.40 ± 0.06
SN	2.26 ± 0.30	1.62 ± 0.21	1.66 ± 0.16	1.71 ± 0.21	1.27 ± 0.13	1.02 ± 0.15	1.04 ± 0.09	1.04 ± 0.90	1.50 ± 0.14	1.45 ± 0.28	1.65 ± 0.34	1.06 ± 0.08
VTA	3.48 ± 1.47	6.45 ± 2.26	3.14 ± 1.08	4.80 ± 1.50	2.15 ± 0.59	2.40 ± 0.48	1.91 ± 0.43	2.12 ± 0.37	1.84 ± 0.20	2.36 ± 0.30	1.91 ± 0.13	1.84 ± 0.20
DR	1.83 ± 0.26	1.97 ± 0.33	1.75 ± 0.26	1.71 ± 0.26	10.14 ± 1.09	9.61 ± 0.45	9.55 ± 0.83	8.20 ± 0.88	0.63 ± 0.07	0.49 ± 0.08	0.46 ± 0.06	0.58 ± 0.10
LC	2.34 ± 0.63	2.14 ± 0.29	2.12 ± 0.40	2.97 ± 0.25	3.63 ± 1.03	4.41 ± 0.64	3.02 ± 0.60	4.25 ± 0.71	1.40 ± 0.21	1.39 ± 0.19	1.48 ± 0.22	1.95 ± 0.44

All data expressed as pg/μg protein and are mean ± SEM, $n = 6$–8 animals/group. All post hoc comparisons are vs. vehicle-treated animals. FC, frontal cortex; ACB, nucleus accumbens; ST, striatum; AMYG, amygdala; VHIPP, ventral hippocampus; SN, substantia nigra; VTA, ventral tegmental area; DR, dorsal raphe nucleus; and LC, locus coeruleus.

[a] Ln transformed.

[*] $p < 0.05$.

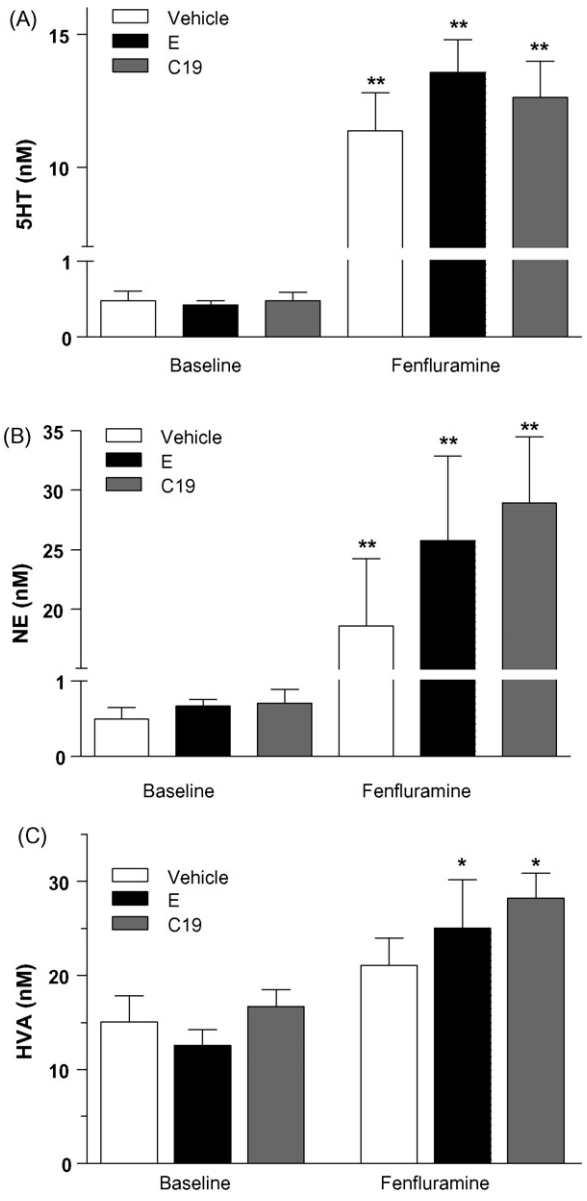

Fig. 3. Effect of ER ligands on monoamines in the ventral hippocampus. Animals ($n = 5$–6/group) were dosed s.c. for 4 days with vehicle, E (0.05 mg/kg) or the ERβ subtype-selective agonist, C19 (3 mg/kg). On Day 4, baseline levels of monoamines and metabolites, determined using microdialysis, did not differ among groups. Mean ± SEM 5HT (A) and NE (B) levels were significantly increased by fenfluramine (10 mg/kg, i.p.) in all three treatment groups whereas HVA (C) was elevated in E- and C19-treated animals. $^{**}p < 0.001$; $^{*}p < 0.05$ vs. baseline.

that activation of either ERα or ERβ may produce a similar response on NE levels. The effects of ERβ activation on NE levels are further supported by the observation that E and the ERβ agonist appeared to affect NE levels in the ventral hippocampus acutely following fenfluramine challenge, although the changes were not statistically different compared to controls.

Both E and PPT also increased tissue levels of NE within the frontal cortex and there was a trend for an increase in NE in this region in animals treated with the ERβ agonist C19. Previous work [51] supports our finding of an E-induced increase in NE in frontal cortex. However, another report shows that long-term treatment with E decreased cortical levels of NE, as well as other monoamines [5], which may be explained by the observation that at least some of the effects of E on monoamines are dependent on the length of steroid exposure [52].

The ER ligand-induced increases of NE in hippocampus and cortex may reflect increased synthesis and/or accumulation of the neurotransmitter in the nerve terminals in these areas. Both ERs are expressed in noradrenergic cell groups in the ventrolateral medulla and nucleus of the solitary tract [25,30]. However, these cell groups send few, if any, projections to hippocampus and cortex and therefore are not likely to have contributed to the increased NE observed in these areas. Instead, both forebrain regions receive projections from noradrenergic neurons of the locus coeruleus [44,53]. Estradiol increases expression of the two enzymes required for synthesis of NE within the locus coeruleus, an effect that may be both time- and dose-dependent [9,54]. Furthermore, ERα is reported to interact directly with the promoter region of at least one of the enzymes [9]. Neurons within the locus coeruleus express ERα and ERβ [25], providing a direct pathway by which ER activation may affect NE synthesis. Importantly, NE activity in the frontal cortex and hippocampus, transmitted almost solely by neurons of the locus coeruleus, is implicated in modulation of cognitive processes (reviewed in [55]).

Levels of the NE metabolite MHPG were altered in two areas of the brain by the two ER subtype-selective agonists but in a region-dependent manner and this also was the case for the DA metabolite DOPAC. Differences in the amount of the DA metabolite HVA released in the ventral hippocampus following fenfluramine administration also were noted in animals treated with E or the ERβ agonist compared to vehicle-treated animals. The catecholaminergic neurotransmitters are metabolized by the enzymes monoamine oxidase and catechol-o-methyl transferase, and activity levels of both are modulated by E [11,13]. While little is known about the role the different ER isoforms play in regulating these enzymes, our data indicate that both receptors may modulate these key metabolic pathways. The majority of monoamine metabolism is thought to occur following reuptake of the neurotransmitters into the cells in which the neurotransmitters were originally synthesized. Thus, the potential modulatory effects of ER ligands on reuptake mechanisms also must be considered. Recent work by Le Saux and Di Paolo [56] showed that E and an ERβ agonist but not ERα agonist increased levels of the DA transporter in striatum. Additional studies are needed to determine more precisely the mechanism(s) through which ERα/β activation influences catecholamine levels in the rat brain.

In the current study, E did not affect levels of 5HT per se but increased levels of the 5HT metabolite 5HIAA in several regions of the CNS. The lack of significant change in 5HT within forebrain areas is consistent with published work [57]. However, recent reports show that E can regulate expression of the 5HT biosynthetic enzyme tryptophan hydroxylase 2 (TPH2) in discrete regions of the rat dorsal raphe, a primary source of forebrain 5HT, and this effect can be mediated through the ERβ [10,58]. It is possible that changes in TPH2 within the dorsal raphe do not translate into measurable differences in 5HT in the forebrain areas examined in our study or that differences in the length of exposure to ER ligands contributes to the differences in findings [3]. In contrast, levels of 5HIAA were increased by ~38% in response to E in the striatum, ventral hippocampus and ventral tegmental area. Increased metabolism of 5HT to 5HIAA may be the result of increased 5HT reuptake into cells via the serotonin reuptake transporter (SERT) or increased activity of monoamine oxidase, as previously mentioned. Indeed, E has been shown to increase SERT in some areas of the female rat brain including in the hippocampus [59], which could result in increased reuptake and metabolism of the indolamine.

Surprisingly, neither of the ER subtype-specific agonists significantly influenced levels of 5HIAA. Inherent variability in monoaminergic measurements in some of the areas e.g., striatum, may have precluded our ability to identify effects of the ER selective ligands. Alternatively, activation of both receptors may be required to induce a significant change in 5HT metabolism. Another intriguing possibility is that E exerted its effects in these areas via the recently identified extra-nuclear ER known as GPR30. GPR30 is expressed throughout the rodent brain [60,61] and it was recently shown to decrease function of the 5HT1A autoreceptor in the rat hypothalamus [62]. Desensitization of the 5HT1A receptor is thought to lead to an increase in 5HT release and theoretically metabolism, however, additional work is needed to understand the role E plays in regulating activity of this novel ER.

4.1. Specificity of subtype-selective ER ligands

The two subtype-selective ER ligands had different effects on body and uterine weight. PPT both increased uterine weight and maintained body weight at a level similar to E, which is consistent with the idea that the ERα plays the predominant role in uterine proliferation and maintenance of body weight [63]. The ERβ agonist C19 is a novel non-steroidal ligand that has low nM affinity for the ERβ and is >70-fold selective for ERβ compared to the ERα [39]. The fact that the ERβ ligand had no effect on uterine or body weight but affected levels of neurotransmitters supports the idea that the dose was sufficient to selectively activate the ERβ. In addition, we have recent data to suggest that C19 improves performance in specific memory tasks similar to the ERβ agonist, DPN [50]. Importantly, the data reflect only one time point and, as mentioned previously, at least some of the effects of E on monoamines are time-dependent [52]. In light of this, it is possible that the effects of ER subtype-selective activation on monoamine concentrations also may vary depending on length of exposure.

4.2. Physiological relevance

Although the impact of ER isoform-selective changes in monoamines on CNS-driven physiology and behavior is unclear, numerous reports support the idea that E affects various dimensions of cognitive and affective function (reviewed in [7,8]). In mice, disruption of the ERβ impaired spatial learning, and augmented aggressive and anxiety-like behavior [64–68]. In ovx rats, an ERβ subtype-selective agonist, but not an ERα selective agonist, improved performance in a hippocampal-dependent memory task [69]. Selective activation of the ERα or ERβ using a 4-day treatment paradigm similar to ours differentially influenced anxiety-like behaviors [70]. Those authors suggest that ERβ activation is anxiolytic whereas ERα activation results in anxiogenic responses in female rats. Other studies that employed acute administration (i.e., 10–60 min) of selective ER agonists directly into the hippocampus support the view that ERβ activation reduces anxiety and increases the extinction rate of a contextual fear memory, but also implicates a non-genomic mechanism of action following activation of the ERβ [71,72].

5. Conclusion

In summary, our data are the first to demonstrate the effects of isoform selective ER activation on tissue concentrations of monoamines and their metabolites in the rat CNS. The data show that activation of either ER isoform with selective agonists influences levels of the catecholamines and/or their metabolites in region- and receptor-specific manners. Activation of either ER produces similar effects on levels of NE in areas critical for cognitive and affective function, such as the frontal cortex and hippocampus. This finding supports the possibility that use of an ERβ subtype-selective agonist may be effective in enhancing neural function without the potential for adverse effects on breast and uterus that are associated with ERα activation.

Conflict of interest statement

L.S.L., P.T.Z., S.E.A., L.C., D.S.L and G.J.H were employed by Merck & Co., Inc. at the time the work was conducted. Other authors have no potential conflicts to disclose.

Acknowledgments

The authors thank Ms. Margaret Sperow, Kathy Phillips and Jennifer Lewis, and Mr. ZuLiang Yao for excellent technical assistance, and Dr. Dan Holder for guidance on statistical analyses. Funded by Merck & Co., Inc. & SO6 GM60654 to Dr. Victoria Luine.

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