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SIGNALING MECHANISMS INVOLVED IN THE ACUTE EFFECTS OF ESTRADIOL ON 5-HT CLEARANCE

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Abstract

Estradiol was found previously to have an antidepressant-like effect and to block the ability of selective serotonin reuptake inhibitors (SSRIs) to have an antidepressant-like effect. The antidepressant-like effect of estradiol was due to estrogen receptor β (ERβ) and/or GPR30 activation whereas estradiol's blockade of the effect of an SSRI was mediated by ERα. This study focuses on investigating signaling pathways as well as interacting receptors associated with these two effects of estradiol.

In vivo chronoamperometry was used to measure serotonin transporter (SERT) function. The effect of local application of estradiol or selective agonists for ERα (PPT) or ERβ (DPN) into the CA_3 region of the hippocampus of ovariectomized (OVX) rats on 5-hydroxytryptamine (5-HT, serotonin) clearance as well as on the ability of fluvoxamine to slow 5-HT clearance was examined after selective blockade of signaling pathways or that of interacting receptors.

Estradiol- or DPN-induced slowing of 5-HT clearance mediated by ERβ was blocked after inhibition of MAPK/ERK1/2 but not of PI3K/Akt signaling pathways. This effect also involved interactions with TrkB, and IGF-1 receptors. Estradiol's or PPT's inhibition of the fluvoxamine-induced slowing of 5-HT clearance mediated by ERα, was blocked after inhibition of either MAPK/ERK1/2 or PI3K/Akt signaling pathways. This effect involved interactions with the IGF-1 receptor and with the metabotropic glutamate receptor 1 but not with TrkB.

This study illustrates some of the signaling pathways required for the effects of estradiol on SERT function and particularly shows that ER subtypes elicit different as well as common signaling pathways for their actions.

Keywords

estradiol; ER agonist; signaling pathways; serotonin transporter; SSRI

INTRODUCTION

The mechanisms of estradiol (E_2) signaling in the brain are complex, tissue specific and could include independent as well as co-dependent effects through estrogen receptor α

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(ERα), ERβ and GPR30 as well as interactions between ER and other membrane receptors (see Bourque et al., 2012). Two major signaling pathways have been associated with the effects of E_2 in the brain; the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways (Raz et al., 2008). A direct interaction between ERα and PI3K was discovered (Simoncini et al., 2000) and numerous kinase and phosphatases have been implicated in the actions of ERα and ERβ (Levin, 1999), likely mediated through an ER-signaling complex. Receptor specific ligands for ERα or ERβ can activate ERK and Akt in hippocampal and cortical neurons (Wade and Dorsa, 2003, Dominguez et al., 2007, Brinton, 2008).

The plasma membrane localization of ERs in caveolae, microdomains at the plasma membrane that assemble signaling proteins, allows their interaction with PI3K/Akt and MAPK/ERK1/2 pathways. Many ER-scaffold proteins and signaling molecules that are associated with membrane ERs may serve to facilitate activation of kinases by E_2. Caveolin proteins, metabotropic glutamate receptors (mGluRs), G proteins, Src, p85, a regulatory subunit of PI3K, Shc and receptor tyrosine kinases, i.e. insulin-like growth factor-1 receptor (IGF-1R) as well as the BDNF receptor (TrkB) have all been reported to serve as components of complexes of interacting proteins with ERs (Etgen et al., 2001; Quesada and Micevych, 2004; Boulware et al., 2005; Marino et al., 2006). Thus, membrane ERs may use other receptors to initiate cell signaling, including TrkB, IGF-1R and mGluRs.

Most preclinical studies that predict the efficacy or examine the mechanism(s) of action of existing or prospective new antidepressants (ADs) are done using male experimental animals. Blockade of serotonin transporter (SERT) function by selective serotonin reuptake inhibitors (SSRIs) is the initial event that triggers a still not completely understood process that results in clinical improvement in depression. The exploration of the mechanisms involved in the modulation of the SERT by ovarian hormones could lead to a better understanding of the interaction between ovarian steroids and the SERT which could have implications for the treatment of depression in women. Previously, it was reported that local application of 17-β estradiol (E_2) directly into the CA_3 region of the hippocampus of ovariectomized (OVX) rats had two effects: (1) slowing of 5-HT clearance; (2) and inhibition of the ability of fluvoxamine to slow the clearance of 5-HT. Both of these effects were seen originally with systemic administration of estradiol benzoate (EB) (Benmansour et al., 2009). Perhaps most importantly, different types of ERs were involved in the two effects of estradiol. Its antidepressant-like effect (i.e., slowing of 5-HT clearance) was due to activation of ERβ and/or GPR30, as shown by effects of the selective agonists DPN for ERβ and G1 for GPR30. By contrast, its blockade of fluvoxamine's inhibitory effect on 5-HT clearance was mediated by ERα, as shown by the effect of the ERα agonist, PPT (Benmansour et al., 2012).

Two major signaling pathways have been implicated in responses underlying antidepressant effects; the PI3K/Akt/Glycogen synthase kinase-3 signaling pathway and the MAPK/ERK signaling pathway (see Tanis and Duman, 2007; Polter and Li, 2011). Numerous studies have implicated these two pathways in the etiology and treatment of mood disorders. Post-mortem studies indicate that brains of individuals who committed suicide have reduced ERK abundance and activity (Dwivedi et al., 2001, Karege et al., 2007). The MAPK/ERK signaling pathway is another central regulator of behavior in rodent models of depression. The ERK signaling cascade is activated by ADs including fluoxetine (Mercier et al., 2004). Pharmacological manipulations of ERK signaling also affect behavior in models of depression and antidepressant response. Systemic blockade of the MAPK pathway in mice with the MEK inhibitor PD184161 produced depressive-like behavior in the forced swim test, tail suspension test, and learned helplessness paradigms, and blocked the effects of the selective norepinephrine reuptake inhibitor desipramine and fluoxetine in the forced swim

test (Duman et al., 2007). These pathways have become major pharmacological targets for putative neuropsychiatric treatments. The interacting receptors investigated in this study, TrkB, IGF-1R and mGluR have not only been shown to be implicated in the effects of estradiol but have also been shown to be involved in the mechanism(s) of action of ADs (Schmidt and Duman, 2007, Duric and Duman, 2013, Artigas, 2013).

The objective of this study was to investigate the involvement of two signaling pathways: MAPK/ERK1/2 and PI3K/Akt as well as possible interacting membrane receptors (TrkB, IGF-1R and mGluRs) in effects linked to activation of ERα or ERβ. Using in vivo chronoamperometry, specific inhibitors of these signaling pathways and antagonists of interacting receptors were applied locally into the CA_3 region of the hippocampus to evaluate their effect on E_2's or DPN's (ERβ agonist) ability to block the SERT as well as the ability of E_2 and that of PPT (ERα agonist) to interfere with the inhibitory effect of SSRIs on the SERT. We hypothesized that there would be specificity/selectivity in these signaling pathways and/or interacting receptors linked to either ERα and ERβ so as to produce the two distinct effects of estradiol on SERT function. It was found that the MAPK/ERK1/2 pathway and IGF-1R are important in both ERα and ERβ signaling whereas the PI3K/Akt pathway and mGluR1 interaction are only important in ERα signaling but not in ERβ signaling.

METHODS

Animals

Ovariectomized (OVX) rats (Sprague-Dawley; 250-350g, Harlan, Indianapolis, IN) were housed on a 12:12h light/dark cycle with lights on at 0700 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.

In vivo chronoamperometry

This was carried out as described previously (Benmansour et al., 2012)

Animal preparation—Sprague-Dawley OVX rats (250-350g) were anesthetized with chloralose (70/mg/Kg)/urethane (700mg/kg) administered intraperitoneally (ip), after tracheal intubation and placed into a stereotaxic apparatus (David Kopf Instruments). The body temperature of the rat was maintained between 37-38°C using a water-circulating heating pad. Rectal temperature was continuously monitored with a probe attached to an YSI temperature meter. The scalp was incised and reflected and a hole drilled in the skull at the desired coordinates. A small burr hole was drilled over the posterior cortex for placement of Ag/AgCl reference electrode.

Electrode preparation—Carbon fiber electrodes (30 m tip diameter, 95-175 m in length) were coated with Nafion to improve their selectivity, then tested for sensitivity to 5-hydroxyindole acetic acid (5-HIAA, 250 M) and calibrated *in vitro* with 5-HT. Only electrodes displaying a selectivity ratio for 5-HT over 5-HIAA > 1000:1 and a linear response ($r^2 > 0.977$) to 5-HT (0.5-3.0 M) were used.

Micropipette preparation—The carbon fiber electrode was positioned adjacent a 7 barrels micropipette using a micromanipulator and the tip separation determined using a

dissecting microscope and was between 250-350 m. The electrode and the multibarrel micropipette were then attached using sticky wax. Micropipette barrels were filled with 5-HT (200 M, Sigma-Aldrich, St Louis, MO), fluvoxamine (400 M, Sigma-Aldrich, St Louis, MO) [fluvoxamine was always used at 4x the amount of 5-HT applied], and the other hormones or drugs to be tested. All drugs were prepared in 0.1M phosphate buffered saline (PBS) and supplemented with 100 M ascorbic acid. The pH of all solutions was 7.4. All drugs were delivered by pressure ejection in a volume of 20-100nl, using a PLI-100 reproducible pico-injector. The volume of the fluid was determined using a dissection microscope (Nikon SMZ-1) fitted with a reticule eye piece.

Electrochemical recordings—The electrode-pipette assembly was lowered into the CA_3 region of hippocampus (stereotaxic coordinates (mm) anterior-posterior (AP), −4.10 from bregma; medio-lateral (ML), +3.30 from midline; dorsal-ventral (DV), −3.60 from dura; Paxinos and Watson (1986)). Chronoamperometric recordings were started 20-30min after the lowering of the assembly to allow the baseline electrochemical signal to stabilize. High-speed chronoamperometric recordings were made using the Fast-16 system (Quanteon). Oxidation potentials consist of 100msec pulses of +0.55 V versus Ag/AgCl were delivered one per second; the electrode was held at the resting potential of 0.0 V between measurements. Oxidation and reduction currents were digitally integrated during the last 80msec of each 100msec voltage pulse.

The electrode placement was confirmed at the conclusion of the electrochemical recordings. Only rats having recordings made in the CA_3 region of the hippocampus (>95%) were included in the analysis.

Experimental design—Both basal 5-HT clearance and the ability of fluvoxamine to block 5-HT clearance were measured 10min after local administration of PBS, E_2 or ER agonists (as shown in the schematic in Figure 1). Since the ERα agonist, PPT, had no effect on 5-HT clearance but blocked the ability of fluvoxamine to slow clearance, PPT was only tested for the latter effect.

Similarly the ERβ agonist, DPN, blocked 5-HT clearance but did not alter the effects of fluvoxamine, so it was only tested for its effect on 5-HT clearance. In addition only inhibitors that altered one or both of estradiol's effects were examined for their effects with the specific ER agonists. In other words, if inhibition of the signaling pathways or the interacting receptors did not alter the effects of estradiol mediated via either ERα or ERβ, then the effect on the inhibition on responses elicited by selective agonists for ERα or ERβ was not studied. As we found previously that the effects of E_2 and ER agonists are similar at an early time point (1-10min) and later time point (40-60min) (Benmansour et al., 2012), we elected to evaluate effects only at 10min.

Effect on E_2 (or ERβ agonist)–induced slowing of 5-HT Clearance

For these experiments, sufficient 5-HT was ejected to produce a peak amplitude of 0.3-0.5μM (amplitudes of this magnitude are small enough not to lose sensitivity of the recording electrode after multiple applications of 5-HT and large enough to enable the detection of decreases in the signal). After a stable signal was obtained following three applications of 5-HT, the inhibitor (inhibitors of signaling pathways or antagonists for interacting receptors) was applied 15min before the application of E_2 or DPN and 5-HT was applied 10min afterwards (see schematic in Figure 1). The clearance time parameter, T_{80}, the time it takes for the peak signal amplitude to be reduced by 80%, was analyzed from the generated 5-HT signal. Data are expressed as percent change in T_{80} ([T_{80} of signal at 10min /T_{80} of signal before inhibitor application] x100).

Effect on E_2's (or ERα agonists') ability to block fluvoxamine's inhibitory effect on 5-HT clearance

Fluvoxamine's ability to block 5-HT clearance is determined by its ability to increase the T_{80} value. As shown in the schematic in Figure 1, after a stable signal was obtained, the inhibitor was applied 15min before the application of E_2 or PPT and fluvoxamine's effect tested 10min after that. Data are expressed as percent change in T_{80} ([T_{80} of signal post fluvoxamine /T_{80} of signal before inhibitor application] x100).

The inhibitors tested were: specific inhibitors for MAPK/ERK1/2: (1) PD98059 (Alessi et al., 1995), 10μg; (2) U0126 (Favata et al., 1998), 100ng; Specific inhibitors for PI3K/Akt: (1) wortmannin (Stephens et al., 1994), 50ng; (2) LY294,002 (Sanchez-Margálet et al., 1994), 30 g; Specific antagonists: for TrkB, k252a (Deltheil et al., 2008; Benmansour et al., 2008), 10ug, for IGF-1R, JB-1 (Pietrzkowski, 1992), 20 g; for mGluR1, LY367,385 (Eaton et al., 1993), 20 g. PD98059, U0126, wortmannin, LY294002, K252a (Sigma-Aldrich, St Louis, MO), JB-1 (Bachem, Torrance, CA) and LY367,385 (Tocris, Ellisville, MO) were all dissolved in PBS or PBS containing ethanol (less than 0.0001%).

The doses of E_2 (20pmoles) and ER agonists- ERα agonist, PPT (60pmoles) and ERβ agonist, DPN (90pmoles) (Tocris, Ellisville, MO) were selected based on our previous studies (Benmansour et al., 2009, 2012). The doses of inhibitors of signaling pathways and those of interacting receptors were selected in pilot studies. In these studies, dose response curves of inhibitors were carried out and the lowest dose, devoid of any effect on the 5-HT signal by itself, and inducing changes on at least one of the effects of E_2; (i.e., either E_2-induced slowing of 5-HT clearance or E_2's inhibition of fluvoxamine-induced slowing of 5-HT clearance), were selected.

4-12 rats were used for each tested drug in each of the two experimental designs 1) the effects of drugs on 5-HT clearance; 2) effects of drugs on fluvoxamine's blockade of 5-HT clearance.

Statistical analysis

Data were analyzed (SigmaStats, Systat Software Inc, San Jose, CA) by two way analysis of variance (ANOVA) followed by Dunnett's analysis for comparisons with the control groups or with the estradiol, DPN or PPT alone groups. Only when there was a significant main effect and/or interaction effect in the ANOVA's were post-hoc analyses carried out. Significance was determined at $p < 0.05$.

RESULTS

1-Effects of inhibitors of signaling pathways on estradiol's effects on SERT function

Two important signaling pathways, MAPK and PI3K have been shown to be associated with actions of estradiol in brain. The involvement of these signaling pathways in E_2's-induced slowing of serotonin clearance or on the ability of E_2 to block fluvoxamine's inhibition of serotonin clearance was examined.

a- Role of MAPK/ERK1/2—As described in the methods section, two selective inhibitors of MAPK/ERK1/2: PD98059 (10 g) and U0126 (100ng) were tested. The inhibitors were applied locally into the CA_3 region of the hippocampus 15min prior to the application of estradiol (20pmol) to evaluate their effect on E_2's ability to block the SERT as well as their ability to interfere with the inhibitory effect of fluvoxamine on SERT function. None of the inhibitors alone had any effect on 5-HT clearance or on fluvoxamine's ability to slow clearance (Figure 2). Local application of PD98059, or U0126 15min prior to E_2, blocked

E_2's ability to increase the T_{80} clearance time parameter as E_2+PD98059 and E_2+U0126 were significantly different from the E_2 alone group (Figure 2a). In addition, these inhibitors restored the ability of fluvoxamine to slow 5-HT clearance, as shown by the increased T_{80} value in the presence of fluvoxamine (Figure 2b). Similar results were obtained when using the selective ER agonists as PD98059 blocked DPN's-induced slowing of 5-HT clearance and PPT's ability to inhibit the fluvoxamine-induced slowing of 5-HT clearance (Tables 1 and 2). Thus, the MAPK pathway seems to be important in these two effects of estradiol mediated by ERα and ERβ.

b- Role of PI3k/Akt—Local application of selective PI3K/Akt inhibitors, wortmannin (50ng) or LY294,002 (30μg), did not alter the ability of E_2 to increase the T_{80} value as a result of the slowing of 5-HT clearance (Figure 3a), but they blocked E_2's inhibition of fluvoxamine's ability to increase the T_{80} value as shown by significant differences between E_2 + PI3K/Akt inhibitor and the E_2 alone group (Figure 3b). Consistent with this, wortmannin also blocked PPT's ability to inhibit the effect of fluvoxamine, as shown by the increased T_{80} after fluvoxamine in the presence of both wortmannin and PPT (Table 2). These data indicate that the PI3K pathway might be important in the effect of E_2 mediated only via ERα activation. None of the inhibitors alone had any effect on 5-HT clearance or on fluvoxamine's ability to slow clearance (Figure 3).

2-Effects of antagonism of interacting receptors on estradiol's effects on SERT function

E_2 activates intracellular pathways by binding to ERs which in turn could engage interactions with cell surface receptors for other compounds such as the BDNF receptor, TrkB, the insulin-like growth factor receptor, IGF-1R, and metabotropic glutamate receptors, mGluRs (Meitzen and Mermelstein, 2011; Arevalo et al., 2012). Pharmacological blockade of possible interacting receptors was used to assess their involvement in the effects of estradiol on SERT function.

As in the experiment described above, the antagonists were applied locally into the CA_3 region of the hippocampus 15min prior to the application of estradiol (20pmoles) to evaluate their effect on estrogen's ability to block the SERT as well as its ability to interfere with the inhibitory effect of fluvoxamine on SERT function (Figure 1). None of the antagonists by itself had any effect on SERT function (Figure 4). Blockade of TrkB by K252a (10μg) blocked the increase in the T_{80} value caused by E_2 whereas it did not restore the ability of fluvoxamine to slow clearance (Figures 4a, 4b). Consistent with this, the DPN-induced slowing of 5-HT clearance was also blocked after TrkB inhibition with K252a (Table 1). Thus, these results indicate that the effects of estradiol mediated by ERβ could involve TrkB activation.

Blockade of IGF-1R with JB1 (20μg) blocked both effects of E_2, as E_2 no longer increased the T_{80} value and fluvoxamine's ability to increase T_{80} was restored (Figures 4a, 4b). Similarly it also blocked the effect of DPN on 5-HT clearance and the effect of PPT on fluvoxamine's ability to slow 5-HT clearance (Tables 1 and 2). Thus, the IGF-1R is implicated in effects of E_2 mediated by both ERα and ERβ.

Pretreatment with a selective antagonist of mGluR1a, LY367385 (20μg), blocked the ability of E_2 and that of the ERα agonist, PPT, to inhibit the fluvoxamine-induced slowing of 5-HT clearance but had no effect on the E_2-increased T_{80} value (Figures 4a, 4b, table 2) therefore indicating a possible interaction between ERα and the mGluR1 in the effects of E_2 on SERT function.

DISCUSSION

These results demonstrate that the effects of E_2 on SERT function are mediated by different as well as common signaling pathways. The E_2-induced slowing of serotonin clearance via activation of ERβ required MAPK/ERK1/2 signaling pathways and involved interactions both with TrkB and IGF-1R. The E_2-induced prevention of the ability of fluvoxamine to slow serotonin clearance, via activation of ERα, required MAPK/ERK1/2 and PI3K/Akt signaling pathways as well as interactions with both IGF-1R and mGluR1.

Rapid activation of both MAPK and PI3K by E_2 has been shown to be important in the effects of estradiol in brain. In neocortical explants, the effects of E_2 on neuronal differentiation were associated with increased ERK phosphorylation (Nethrapalli et al., 2001). It was shown that the MAPK cascade plays a central role in E_2-induced structural plasticity in cholinergic neurons (Dominguez et al., 2004). E_2 also induces rapid activation of ERK in primary cultures of astroglia (Ivanova et al., 2001) and in explants of cerebral cortex (Toran-Allerand et al., 1999, Singh et al., 2000, Setalo et al., 2002). In the rat hippocampus, E_2's activation of MAPK/ERK1/2 protects against quinolone-induced toxicity (Kuroki et al., 2001). Activation of ERK was detected *in vivo* in the hypothalamus after systemic administration of estradiol (Cardona-Gomez et al., 2002). Consistent with such observations, we found that inhibition of MAPK/ERK1/2 blocked the ability of E_2 to slow 5-HT clearance and blocked the ability of E_2 to prevent the effect of fluvoxamine on 5-HT clearance (Figure 2a and b).

The PI3K/Akt pathway is involved in a wide array of neuronal functions, including cell survival and long term potentiation (LTP) (Sanna et al., 2002; Sui et al., 2008). Estradiol prevented injury-induced apoptosis via activation of Akt (Wilson et al., 2002). In the rat retina, activation of the PI3K pathway is important in E_2-mediated protection against light-induced photoreceptor degeneration (Yu et al., 2004). We also found E_2's activation of PI3K/Akt to be necessary for the effect of the hormone in blocking the ability of fluvoxamine to slow 5-HT clearance (Figure 3b). However, inhibition of PI3K/Akt did not alter the ability of E_2 to slow 5-HT clearance (Figure 3a). Effects of estradiol can be mediated by the activation of both MAPK/ERK and PI3K/Akt. For example, phosphorylation of mTOR in the dorsal hippocampus, necessary for E_2 -enhanced object recognition and memory consolidation, is dependent on upstream activation of both ERK and PI3K signaling pathways (Fortress et al., 2013). Similarly, we found E_2's inhibition of the effect of fluvoxamine also to be dependent on the activation of both MAPK/ERK1/2 and PI3K/Akt signaling (Figures 2b and 3b). By contrast, E_2-induced slowing of 5-HT clearance was dependent only on MAPK/ERK1/2 and not on PI3K/Akt signaling (Figures 2a and 3a). Similarly, the effect of an ERα agonist was dependent on the activation of both MAPK/ERK1/2 and PI3K signaling pathways whereas those of the ERβ agonist involved the MAPK/ERK1/2 but not PI3K/Akt pathways (Tables 1 and 2). Consistent with these data, it has been shown that ERα is involved in the activation of PI3K/Akt whereas ERβ is not (Kahlert et al., 2000; Mendez et al., 2003; 2005). Recently estradiol administration was shown to increase immunoreactivity for phosphorylated Akt at 6h and that of phosphorylated TrkB at 48h after its administration in the hippocampus of mice. These effects were abolished in ERα and ERβ knockout mice and therefore require both ERα and ERβ activation. These results indicate that ERα and ERβ do not always have distinct effects; they can have complementary roles as they may activate distinct signaling pathways that converge onto common downstream targets (Spencer-Segal et al., 2012).

Estrogen and BDNF seem to share common targets, effects, and mechanisms of action in the hippocampus (Scharfman and Maclusky, 2005; 2006). The action of BDNF is mediated through its high affinity tyrosine kinase receptor B (TrkB). Blockade of TrkB prevented the

E_2-induced slowing of 5-HT clearance but had no effect on the ability of E_2 to prevent the effect of fluvoxamine (Figures 4a and 4b). Further evidence was obtained with the ERβ agonist, as DPN no longer slowed the clearance of 5-HT after blockade of TrkB with K252a (Table 1). BDNF has been implicated in the mechanism(s) of action of ADs (see Schmidt and Duman, 2007). Chronic treatment with ADs upregulate the expression of BDNF and TrkB in the hippocampus and cortex; in addition, direct infusion of BDNF into the hippocampus or into cerebral ventricles produces AD-like effects in two animal models of depression (see Schmidt and Duman, 2007). Evidence that activation of the TrkB receptor is required for a behavioral response typically induced by ADs has been shown as well (Saarelainen et al., 2003)**.** Although upregulation of BDNF expression is observed only after chronic AD drug treatments, there is also evidence that acute AD treatment activates TrkB receptors (Saarelainen et al., 2003; Rantamaki et al., 2007; Furmaga et al., 2012). Thus, BDNF could act as an intermediate signaling molecule in the antidepressant-like effect of estradiol mediated by ERβ.

The interaction of estradiol and IGF-1R in neuroprotection has been characterized in experimental models of excitotoxicity, Parkinson's disease and stroke (Jover-Mengual et al., 2007; Quesada et al., 2008; Lebesgue et al., 2009; Garcia-Segura et al., 2010; Selvamani and Sohrabji, 2010). It was shown that the inhibition of brain IGF-1 signaling in these models resulted in attenuation of the neuroprotective effects of estradiol. Blockade of IGF-1R with JB-1, a selective competitive antagonist of IGF-1 autophosphorylation, attenuated the neuroprotective effects of both E_2 and IGF-1, suggesting that the neuroprotective actions of E_2 depend on the co-activation of both ERs and IGF-IR (Quesada and Micevych, 2004). Moreover, intracerebroventricular infusion of JB-1 inhibits the estrogen-induced LH surge and sexual behavior in ovariectomized rats (Quesada and Etgen, 2002). In addition to interactions between ERs and IGF-1R, IGF-1 has been implicated in the mechanism of action of antidepressants, perhaps through a mechanism involving serotonin. For example, chronic administration of fluoxetine to rats increased protein levels of IGF-1 in hippocampus (Khawaja et al., 2004). Acute intraventricular delivery of IGF-1 to mice had an antidepressant-like effect in the tail suspension test (Malberg et al., 2007). IGF-1 induced AD-like effects in the FST three days after its intraventricular administration and this effect required the presence of 5-HT (Hoshaw et al., 2008). We found that antagonism of the IGF-1R with JB-1 blocked both E_2- or DPN-induced slowing of 5-HT clearance and the ability of either E_2 or PPT to prevent the inhibitory effect of fluvoxamine on 5-HT clearance (Figures 4a and 4b, Tables 1 and 2). Collectively, these findings support functional interactions between ER subtypes and IGF-1R in the effects of estradiol on SERT function. These interactions could be complex as it was shown in neurons that estradiol induces incorporation of ERα into a macromolecular complex in which IGF-1R and several components of IGF-1R signaling are associated and lead to the regulation of neuroprotection and improvement of brain homeostasis (Garcia-Segura et al., 2010; Alonso and Gonzalez, 2012).

In addition to ER cross-talk with IGF-1 receptors, there is evidence for interaction between ER and metabotropic glutamate receptor (mGluRs) signaling in hippocampal, hypothalamic and striatal neurons (Boulware et al., 2005; Dewing et al., 2007; Micevych and Mermelstein, 2008; Grove-Strawser et al., 2010). Estradiol binding to the ER promotes transactivation of mGluR, initiating mGluR signaling without the need for glutamate (Boulware et al., 2005; Dewing et al., 2007; Kuo et al., 2010). Such a receptor–receptor interaction of ERs and mGluRs is supported by co-immunoprecipitation experiments that indicate, for example, that ERα can directly interact with mGluR1a (Dewing et al., 2007). The ER/mGluR interactions are dependent upon caveolin (CAV) proteins that are essential for the trafficking and clustering of signaling molecules (Boulware et al., 2007). Interestingly, there is a brain region specific ER–CAV interaction (Meitzen and Mermelstein, 2011). In hippocampal

neurons, ERα interaction with either mGluR1 or mGluR2/3 was dependent upon CAV3 or CAV1, respectively. Conversely, ERβ interacts with mGluR2/3 via CAV3 (Boulware et al., 2007). In striatal neurons, ERα via CAV1 activates mGluR5 (Grove-Strawser et al., 2010). We found that an interaction between ERα and mGluR1a seems to be important in the effects of estradiol on SERT function. Antagonism of mGluR1a blocked the ability of E_2 and that of the ERα agonist PPT to inhibit the effects of fluvoxamine but did not alter E_2's ability to slow 5-HT clearance, implying that ERβ does not require an interaction with this receptor (Figures 4a and 4b, Table 2). It is not known which of the caveolin proteins are associated with the effect of estradiol mediated by ERα on SERT function. It is also worth noting that a role for mGluR in anxiety and depression models has been shown and that several mGluR selective agents are described to modulate stress, anxiety and depressive behavior (See Artigas, 2013).

E_2 activation MAPK/ERK1/2 and PI3K/Akt signaling pathways involve not only ERs but also interactions with other receptors. Given the similarity of the intracellular signaling pathways activated by ER with those activated by mGluR, IGF-1R and TrkB, it is possible that the interacting receptors may serve to facilitate activation of kinases by E_2.

At this time, it is unclear how the ER-induced activation of the signaling pathways in question lead to the alterations observed in SERT function. SERT proteins exhibit posttranslational regulation (Steiner et al., 2008). Serotonin transporter regulation can occur via phosphorylation dependent and independent posttranslational modifications (Ramamoorthy et al., 2011). In human embryonic kidney cells, protein kinase C (PKC) activation and PP2A inhibition trigger SERT phosphorylation correlated with decreased serotonin uptake function that occurs with a loss of SERT surface density (Qian et al., 1997; Ramamoorthy et al., 1998; Ramamoorthy and Blakely, 1999). Supporting an acute SERT regulation, adenosine A3 receptor stimulation increased SERT uptake function in rat basophilic leukemia (RBL-2H3) cells and mouse mid-brain and hippocampal synaptosomes. This effect is thought to be mediated by both a PKG-dependent surface density increase of the SERT, and p38-MAPK dependent activation of SERT intrinsic activity (Samuvel et al., 2005; Zhu et al., 2005). Multiple signaling pathways seem to contribute to the regulation of SERT mediated 5-HT clearance (Blakely et al., 2005). It is therefore possible that the downstream signaling pathways of estradiol could induce SERT phosphorylation and therefore decrease its uptake function. Future studies are needed to delineate the changes occurring in SERT (i.e. phosphorylation, internalization or conformational changes) and that would account for the effects of estradiol.

In conclusion, as shown previously (Benmansour et al., 2009; 2012), E_2 has two opposing effects with respect to the functioning of the SERT: one is to inhibit the SERT so as to produce an antidepressant-like effect but the other is to inhibit the ability of SSRIs to block the SERT. These two effects are mediated by different types of ERs interacting with similar as well as different receptors and using different as well common signaling pathways. Targeting the ER subtypes and/or their interacting receptors/ signaling pathways may reveal a strategy to permit only beneficial effects of estrogen without its deleterious effect on SSRI-efficacy. Further delineating the signaling mechanisms involved in the effects of estradiol on SERT function could lead to a better understanding of the effects of endogenous and exogenous estrogens on mood.

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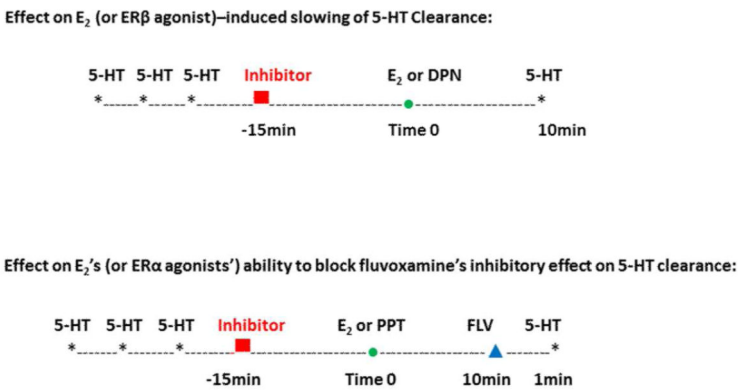

Figure 1.

Schematic showing the experimental design used for local administration of estradiol, DPN or PPT and an inhibitor to study effects on 5-HT clearance or effects on fluvoxamine ability to alter 5-HT clearance.

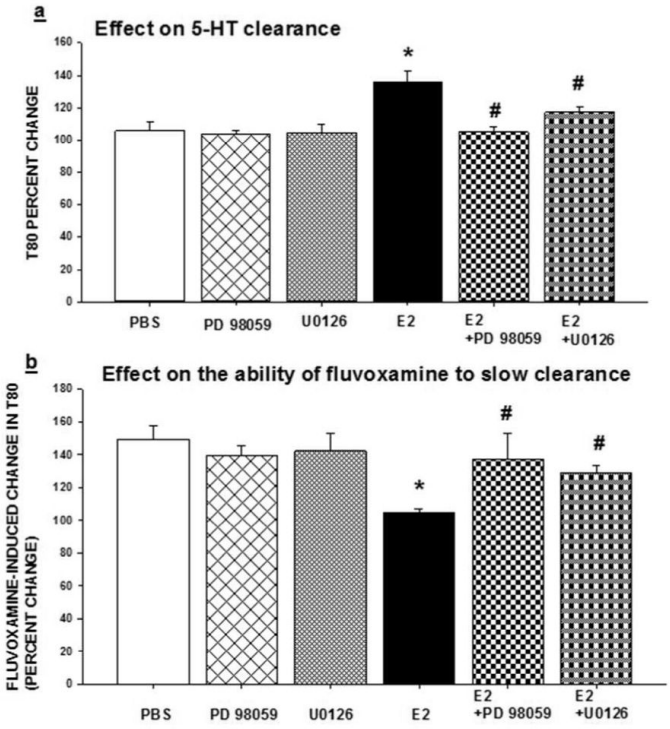

Figure 2.

Effects of inhibitors of MAPK/ERK1/2 on estradiol's effects on SERT function. Selective inhibitors of MEK1/2, PD98059 (10μg) or U0126 (100ng), were applied locally into the CA3 region of the hippocampus 15min prior to local application of 17-β-estradiol (E_2; 20 pmol), and their effects on:

(a) The clearance time parameter T_{80} were measured at 10 min post E_2 administration. Bar and brackets represent the T_{80} value as a percentage of the pretreatment value ± SEM (n = 4–8); Two way analysis of variance was carried out for the percent change in T_{80} values for E_2 and/or the inhibitors. There was a significant main effect for inhibitor [$F(2,30)=6.715$, $p=0.004$] and for E_2 [$F(1,30)=14.879$, $p<0.001$] as well as a significant interaction between inhibitor x E_2 [$F(2,30)=4.852$, $p=0.015$]. Dunnett's post hoc analysis was carried out. $^{*}p < .001$, comparing E_2 within PBS and $^{\#}p < .01$, comparing value of E_2 with inhibitor to value of E_2 alone.

(b) The ability of fluvoxamine to increase the T_{80} value was measured at 10 min post E_2 administration. Bar and brackets represent the percent change in T_{80} value after fluvoxamine ± SEM (n = 4–8). The data were analyzed by a two-way ANOVA. The percentage change in the T_{80} value produced by fluvoxamine was the parameter analyzed and the effect of E_2 in the absence or presence of the inhibitors was evaluated. There was no significant main effect for inhibitor [$F(2,31)=2.051$, $p=0.146$] but there was a significant main effect for E_2 [$F(1,31)=16.398$, $p<0.001$] as well as a significant interaction between inhibitor x E_2 [$F(2,31)=6.934$, $p=0.003$]. Dunnett's post hoc analysis was carried out. $^{*}p < .001$, comparing E_2 within PBS and $^{\#}p < .01$, comparing value of E_2 with inhibitor to value of E_2 alone.

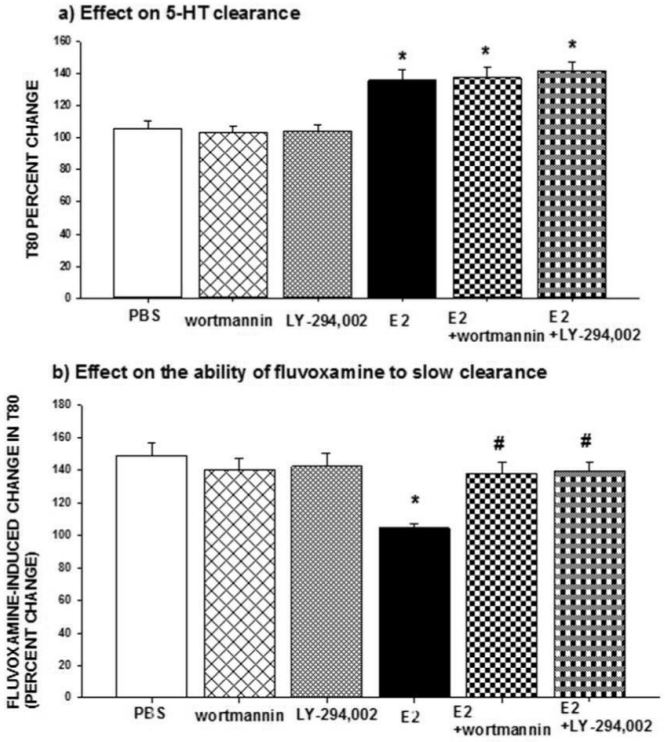

Figure 3.

Effects of inhibitors of PI3K/Akt on estradiol's effects on SERT function. Selective inhibitors of PI3K/Akt, wortmannin (50ng) or LY294,002 (30μg), were applied locally into the CA3 region of the hippocampus 15min prior to local application of 17-β-estradiol (E_2; 20 pmol), and their effects on:

(a) The clearance time parameter T_{80} were measured at 10 min post E_2 administration. Bar and brackets represent the T_{80} value as a percentage of the pretreatment value ± SEM (n = 5–7); Two way analysis of variance was carried out for the percent change in T_{80} values for E_2 and/or inhibitors. There was no significant main effect for inhibitor [$F(2,29)=0.148$, $p=0.863$] but there was a significant main effect for E_2 [$F(1,29)=56.982$, *$p<0.001$] and no significant interaction between inhibitor x E_2 [$F(2,29)=0.258$, $p=0.774$].

(b) The ability of fluvoxamine to increase the T_{80} value was measured at 10 min post E_2 administration. Bar and brackets represent the percent change in T_{80} value after fluvoxamine ± SEM (n = 4–12). The data were analyzed by a two-way ANOVA. The percentage change in the T_{80} value produced by fluvoxamine was the parameter analyzed and the effect of E_2 in the absence or presence of the inhibitors was evaluated. There was a significant main effect for inhibitor [$F(2,34)=3.114$, $p=0.05$] and for E_2 [$F(1,34)=9.759$, $p=0.004$] as well as a significant interaction between inhibitor x E_2 [$F(2,34)=7.782$, $p=0.002$]. Dunnett's post hoc analysis was carried out. *$p < .001$, comparing E_2 within PBS and $^{\#}p < .001$, comparing value of E_2 with inhibitor to value of E_2 alone.

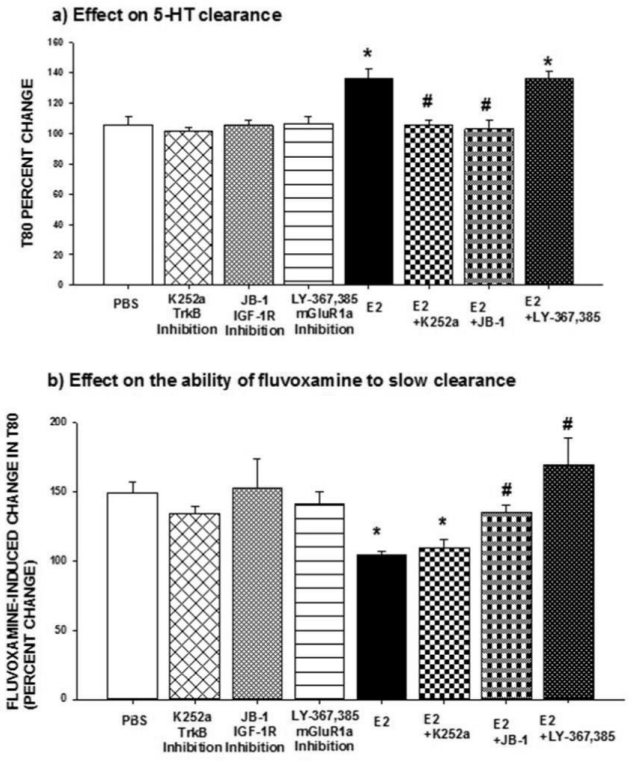

Figure 4.

Effects of antagonists of interacting receptors on estradiol's effects on SERT function. Selective antagonists of TrkB, k252a (10μg); of IGF-1R, JB1(20μg); or that of mGluR1a, LY367385 (20μg) were applied locally into the CA3 region of the hippocampus 15min prior to local application of 17-β-estradiol (E_2; 20 pmol), and their effects on:

(a) The clearance time parameter T_{80} were measured at 10 min post E_2 administration. Bar and brackets represent the T_{80} value as a percentage of the pretreatment value ± SEM (n = 5–11); Two way analysis of variance was carried out for the percent change in T_{80} values for the E_2 and/or inhibitors. There was a significant main effect for inhibitor [$F(3,52)=6.604$, $p<0.001$] and for E_2 [$F(1,52)=15.400$, $p<0.001$] as well as a significant interaction between inhibitor x E_2 [$F(3,52)=4.903$, $p=0.004$]. Dunnett's post hoc analysis was carried out. *p < .001, comparing value of E with inhibitor to value of E # 2 2 alone and p < .001, comparing value of E_2 with inhibitor to value of E_2 alone.

(b) the ability of fluvoxamine to increase T_{80} were measured at 10 min post E_2 administration. Bar and brackets represent the percent change in T_{80} value after fluvoxamine ± SEM (n = 4–10).

The data were analyzed by a two-way ANOVA. The percentage change in the T_{80} value produced by fluvoxamine was the parameter analyzed and the effect of E_2 in the absence or presence of the inhibitors was evaluated. There was a significant main effect for inhibitor [$F(3,45)=4.365$, $p=0.009$] and for E_2 [$F(1,45)=4.215$, $p=0.046$] as well as a significant interaction between inhibitor x E_2 [$F(3,45)=4.275$, $p=0.010$]. Dunnett's post hoc analysis was carried out. *p < .001, comparing E_2 within PBS and $^{\#}p$ < .05, comparing value of E_2 with inhibitor to value of E_2 alone.

Table 1

Effects of inhibition of signaling pathways and that of interacting receptors on the DPN-increased T_{80} value

	Vehicle + vehicle	**Vehicle + DPN**	**PD98059 + DPN**	**K252a + DPN**	**JB-1+ DPN**
T_{80} % change	106.0 ±5.04	149.5* ±9.61	103.1# ±6.00	96.7# ±6.05	103.1# ±7.02

[a] Mean of T_{80} value expressed as a percentage of pretreatment value ±SEM of 5-7 rats

Two way analysis of variance was carried out for the percent change in T_{80} values for DPN and/or the inhibitors. Inhibitors alone, not shown in this table did not change T_{80} values (as shown in Figures 2a and 4a). The data from the inhibitors alone were included in the statistical analyses. There was a significant main effect for inhibitor [$F(3,41)=14.122$, $p<0.001$] and for DPN [$F(1,41)=7.522$, $p=0.009$], as well as a significant interaction between inhibitor x DPN [$F(3,41)=12.984$, $p<0.001$]. Dunnett's post hoc analysis was carried out.

* $p < .001$, comparing DPN within PBS and

# $p < .001$, comparing value of DPN with inhibitor to value of DPN alone.

Table 2

Effects of inhibition of signaling pathways and that of interacting receptors on PPT's blockade of the fluvoxamine-induced increase in the T_{80} value

	Fluvoxamine+ vehicle+ vehicle	**Fluvoxamine+ vehicle+ PPT**	**Fluvoxamine+ PD98059+ PPT**	**Fluvoxamine+ wortmannin+ PPT**	**Fluvoxamine+ JB-1+ PPT**	**Fluvoxamine+ LY367,385+ PPT**
T_{80} % change	149.3[a] ±7.80	102.4[*] ±3.66	130.7[#] ±9.04	132.3[#] ±11.06	133.3[#] ±14.01	141.2[#] ±15.00

[a]Mean of percent in T_{80} value after fluvoxamine ±SEM of 4-7 rats

The data were analyzed by Two-way ANOVA. The percentage change in the T_{80} value produced by fluvoxamine was the parameter analyzed and the effect of PPT in the absence or presence of the inhibitors was evaluated. Inhibitors alone, not shown in this table, did not change the fluvoxamine-induced increased T_{80} value (as shown in Figures 2b, 3b and 4b). There was no significant main effect for inhibitor [$F(4,50)=1.554$, $p=0.201$] but there was a significant main effect for PPT [$F(1,50)=11.321$, $p<0.001$] and a significant interaction between inhibitor x PPT [$F(4,50)=3.013$, $p=0.026$]. Dunnett's post hoc analysis was carried out.

[*] $p < .001$, comparing PPT within PBS and

[#] $p < .05$, comparing value of PPT with inhibitor to value of PPT alone.