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# Contribution of estrogen receptor subtypes, ERα, ERβ, and GPER1 in rapid estradiol-mediated enhancement of hippocampal synaptic transmission in mice

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## Abstract

Estradiol rapidly modulates hippocampal synaptic plasticity and synaptic transmission; however, the contribution of the various estrogen receptors to rapid changes in synaptic function is unclear. The current study examined the effect of estrogen receptor selective agonists on hippocampal synaptic transmission in slices obtained from 3-5 month old wild type (WT), estrogen receptor alpha (ERαKO), and beta (ERβKO) knockout female ovariectomized mice. Hippocampal slices were prepared 10-16 days following ovariectomy and extracellular excitatory postsynaptic field potentials were recorded from CA3-CA1 synaptic contacts before and following application of 17β-estradiol-3-benzoate (EB, 100 pM), the G-protein estrogen receptor 1 (GPER1) agonist G1 (100 nM), the ERα selective agonist propyl pyrazole triol (PPT, 100 nM), or the ERβ selective agonist diarylpropionitrile (DPN, 1 μM). Across all groups, EB and G1 increased the synaptic response to a similar extent. Furthermore, prior G1 application occluded the EB mediated enhancement of the synaptic response and the GPER1 antagonist, G15 (100 nM), inhibited the enhancement of the synaptic response induced by EB application. We confirmed that the ERα and ERβ selective agonists (PPT, DPN) had effects on synaptic responses specific to animals that expressed the relevant receptor; however, PPT and DPN produced only a small increase in synaptic transmission relative to EB or the GPER1 agonist. We demonstrate that the increase in synaptic transmission is blocked by inhibition of extracellular signal-regulated kinase (ERK) activity. Furthermore, EB was able to increase ERK activity regardless of genotype. These results suggest that ERK activation and enhancement of synaptic transmission by EB involves multiple estrogen receptor subtypes.

### Keywords

Estrogen; estrogen receptor alpha and beta; GPR30; GPER1; estradiol; G1; G15; hippocampus; synaptic plasticity; ERK

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## 1. Introduction

Estradiol acts on the nuclear estrogen receptors, alpha (ERα) and beta (ERβ) in the hippocampus to regulate genes that are important for neuronal growth, neuroprotection, and the maintenance of memory function (Aenlle and Foster, 2010; Han et al., 2013). One of the ways in which estradiol influences memory is by inducing long-term changes on synaptic plasticity and neurotransmission (Smith and McMahon, 2005). In addition, estradiol can rapidly activate signaling cascades involved in synaptic plasticity via nongenomic actions. Specifically, estradiol rapidly increases synaptic responses mediated by N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Foy et al., 1999; Fugger et al., 2001; Sharrow et al., 2002; Teyler et al., 1980; Wong and Moss, 1992). The increase in synaptic transmission is believed to result from a shift in the balance of kinase/phosphatase activity, including increased activity of the extracellular signal-regulated kinase (ERK) (Bi et al., 2001; Kramar et al., 2009; Sharrow et al., 2002; Tanaka and Sokabe, 2013; Zadran et al., 2009). The rapid effects are thought to result from the activity of membrane-associated estrogen receptors and in many cases are mimicked by receptor selective agonists (Boulware and Mermelstein, 2005; Mukai et al., 2007; Smejkalova and Woolley, 2010; Wu et al., 2011; Zadran et al., 2009; Zhao and Brinton, 2007). Indeed, results from mutant mice in which ERα or ERβ have been knocked out suggest that the estrogen-induced rapid enhancement of synaptic transmission involves multiple estrogen receptors (Foster et al., 2008; Fugger et al., 2001; Gu et al., 1999); however, the contribution of each subtype is unclear.

Interestingly, application of ERα or ERβ antagonists does not block the rapid increase in synaptic transmission and increases glutamate receptor responsiveness in the hippocampus (Fugger et al., 2001; Gu et al., 1999; Moss and Gu, 1999), suggesting involvement of a mechanism independent of ERα and ERβ in mediating rapid effects on synaptic function. The role of estrogen receptors in mediating the rapid increase in ERK activation is less clear with studies indicating that estrogen receptor antagonists inhibit ERK activation, fail to inhibit ERK activation, or induce ERK activation (Dominguez et al., 2007; Kuroki et al., 2000; Singh et al., 1999; Xu et al., 2011; Zhao and Brinton, 2007). A recently described G-protein coupled estrogen receptor (GPER1, formerly known as GPR30) is widely distributed in the brain, with high expression in the hippocampus in association with postsynaptic proteins (Akama et al., 2013; Brailoiu et al., 2007; Hazell et al., 2009; Srivastava and Evans, 2013). Furthermore, GPER1 activation can induce a rapid increase in synaptic transmission in the hippocampus (Lebesgue et al., 2010). Thus, each receptor subtype may contribute to the rapid increase in synaptic transmission.

The current study was designed to parse out the contribution of each estrogen receptor subtype to the rapid enhancement induced by bath application of 17β-estradiol-3-benzoate (EB). For this study, we employed estrogen-receptor-selective pharmacological interventions in wild type (WT), estrogen receptor alpha (ERαKO), and beta (ERβKO) knockout mice (KO). The results indicate that all three receptors contribute to the rapid effects of estradiol on hippocampal synaptic function; however, GPER1 is a major contributor to the rapid increase in synaptic transmission and activation of homodimeric

ERα or ERβ provide only modest modulation of synaptic transmission in CA3-CA1 hippocampal synapses of female mice.

## 2. Materials and Methods

### 2.1. Animals

ERα-/- (ERαKO: n = 26) (Lubahn et al., 1993) and ERβ-/- (ERβKO: n = 30) (Krege et al., 1998) mice were created from heterozygous mouse colonies. The genotypes of the mice were screened using PCR amplification as previously described (Krege et al., 1998; Lubahn et al., 1993). Wild type (WT: n = 46) littermates produced from the ERαKO and ERβKO breeding colonies were combined into one WT group. Animals were housed 3-5 per cage and maintained on 12:12 light:dark cycle (lights on at 6 am).

### 2.2. Surgery

All procedures involving animal subjects have been reviewed and approved by the Institutional Animal Care and Use Committee at the University of Florida and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. All mice received ad lib access to food (Purina mouse chow, St Louis, MO) and water. Surgeries were performed as described previously (Foster et al., 2008; Fugger et al., 2001; Han et al., 2013). Briefly, female mice (3-5 months old) were anesthetized (2 mg ketamine and 0.2 mg xylazine per 20 grams of body weight) and ovaries were removed through a small midline incision on the abdomen. Hippocampal slices were prepared 10-16 days following ovariectomy. The 10-16 day period permitted recovery from surgery and is within a time window in which the hippocampus and hippocampal-dependent behaviors remain responsive to estrogen receptor activation (Aenlle and Foster, 2010; Foster, 2012; Fugger et al., 2001; Han et al., 2013; McLaughlin et al., 2008; Phan et al., 2011).

### 2.3. Hippocampal Slice Preparation

The methods for hippocampal slice preparation have been published previously (Foster et al., 2008; Fugger et al., 2001; Sharrow et al., 2002). Briefly, ovariectomized mice were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ) and swiftly decapitated. The brain was rapidly removed and the hippocampus sliced in the transverse plane into ~400 μm sections using a tissue chopper. The slices were incubated in a holding chamber containing artificial cerebrospinal fluid (aCSF) (NaCl, 124 mM; KCl, 2 mM; $KH_2PO_4$, 1.25 mM; $MgSO_4$, 2.0 mM; $CaCl_2$, 2.0 mM; $NaHCO_3$, 26 mM; glucose, 10 mM) at room temperature for at least 60 min before transferring to recording chamber. The pH was maintained at 7.4 with 95%/5% $O_2/CO_2$. Thirty minutes before recording, 3-4 slices were transferred to an interphase recording chamber (Warner Instrument Corporation, Hamden, CT) and were perfused (2 ml/min) with oxygenated aCSF. The recording was performed at 30±0.5°C (Automatic Temperature Controller, TC-324B, Warner Instrument Corporation, Hamden, CT).

### 2.4. Extracellular Field Potential Recordings

The methods for extracellular recordings from CA3-CA1 hippocampal slices obtained from mice have been previously published (Foster et al., 2008; Fugger et al., 2001; Sharrow et al., 2002). Briefly, extracellular excitatory postsynaptic potentials (EPSPs) were recorded with aCSF-filled glass micropipettes (4-6 MΩ). A recording electrode was localized to the CA1 dendrites in the middle of the stratum radiatum for examination of the CA3-CA1 synaptic responses. A stimulating electrode (outer pole: stainless steel, 200 μm diameter; inner pole: Platinum/Iridium, 25 μm diameter, FHC, Bowdoinham, ME) was positioned on one side of the recording electrode. Biphasic constant current stimulus (100 μsec) was delivered using SD9, Grass stimulators (Grass Technologies, West Warwick, RI) at 0.033 Hz. The signals were amplified 100 times, filtered, and stored on a dedicated computer hard drive for off-line analysis. To measure the influence of genotype on basal synaptic transmission, input-output curves were constructed using a computer program that determined the maximum amplitude of the synaptic response within a window. Following collection of input-output data, the stimulation intensity was set to obtain the half maximum amplitude and stimulus pulse pairs were delivered (50 ms inter-pulse interval). The EPSP slope (mV/msec) was measured as the difference between two cursors, separated by 1 msec, and placed on the middle portion of the descending phase of the EPSP. The paired-pulse facilitation (PPF) ratio was calculated by dividing the slope of the second synaptic response by the slope of the first response. Baseline EPSP slopes were collected for at least 10 min prior to drug application and the response was followed for at least 45 min following drug application. Changes in synaptic strength were quantified as the average percent change from baseline during the 40-45 min following drug application.

### 2.5. Western blot

To examine ERK activation, hippocampal slices (4-5 slices per time point) were exposed to the GPER1 selective antagonist, G15, followed by EB application, or EB application alone, and slices were collected before and at various times following exposure to EB. Slices were frozen in liquid nitrogen prior to storage at −80°C. Tissue was homogenized in RIPA buffer (Thermo Scientific) containing protease and phosphatase inhibitors (Thermo Scientific) and centrifuged at 20000 × g for 30 min at 4°C. The supernatant was collected and protein concentrations were determined using a Pierce BCA protein assay (Thermo Scientific). Aliquots (20 μg/lane) and Kaleidoscope protein standards (Bio-Rad) were separated on 10% resolving gels (Mini-Protean TGX Precast, Bio-Rad). Two methods were used to visualize blots. Blots were visualized using a Western blot protein detection system (Odyssey, LI-COR Bioscience, Lincoln, NE) or blots were exposed to BioMaxMR film (Kodak, Rochester, NY), developed on a film processor (Konica Medical Corp, Ramsey NJ). Protein band density for phosphorylated and total ERK1 (44 kD) and ERK2 (42 kD) were quantified, ratios (pERK/tERK) were calculated, and normalized to the control, the pre-EB application baseline. For a subset of slices, both blot detection techniques were employed and no difference in measures was observed between the techniques.

For film processing, the gels were transferred to PVDF membranes. The immunoblots were blocked in Tris buffered saline with 5% non-fat powdered milk and 0.1% tween-20 for 1 hr at room temperature followed by overnight incubation at 4°C with diluted primary

antibodies pERK1/2 (1:2000, Cell Signaling Technology) or ERK1/2 total (1:10000, Cell Signaling Technology). Subsequently, blots were washed with TBS and secondary antibodies were applied for 1 hr at room temperature. Blots were washed with TBS and an ECL Plus kit (GE Healthcare) was employed for visualization. Blots were scanned using GS-800 Calibrated Densitometer (Bio-Rad), and bands were quantified using ImageJ software (National Institute of Health, Bethesda, MD).

For the Odyssey system, gels were transferred to Odyssey nitrocellulose membranes (LI-COR Bioscience, Lincoln, NE). The immunoblots were blocked in Odyssey Blocking Buffer (product # 927-5000, LI-COR) for 1 hr at room temperature followed by overnight incubation at 4°C with diluted primary antibodies pERK1/2 (1:2000, Cell Signaling Technology), ERK1/2 total (1:5000, Cell Signaling Technology), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:20000, EnCor Biotechnology). IRDye secondary antibodies (Product # 926-68020 and 926-32211, LI-COR) were applied for 1 hr at room temperature. Blots were washed with TBST followed by TBS. Membranes were scanned and quantified using Odyssey Infrared Imaging System and Image Studio Software version 2.0 (LI-COR Bioscience).

### 2.6. Drugs

All drugs were bath applied by addition to the aCSF. Unless indicated, each slice received only one treatment, though several slices were examined from each animal. Thus, effects of different drugs were examined in different slices from the same animal. Furthermore, tests for effects of vehicle (EtOH for EB, PPT, and DPN and DMSO for G1) were interleaved with drug studies. The dose of receptor selective agonists was selected due to previous work suggesting maximal effectiveness of action and minimal interaction with the other estrogen receptors (Rossi et al., 2010; Stauffer et al., 2000). 17β-estradiol-3-benzoate (EB) (100 pM), rather than estradiol, was employed as a positive control in order to be consistent with our previous work examining rapid effects on synaptic function (Foster et al., 2008; Fugger et al., 2001; Sharrow et al., 2002). Propylpyrazole triol (PPT), diarylpropionitrile (DPN), and PD98059 were obtained from Tocris (Tocris Bioscience, Ellisville, MO). EB (Sigma, 100 pM), PPT (100 nM), DPN (1 μM), and G15 (Cayman Chemical, 100 nM) were initially dissolved in a small amount of ethanol and diluted to a final ethanol concentration of 0.001%. G1 (Calbiochem, 100 nM) and PD98059 (20 μM) were initially dissolved in a small amount of dimethyl sulfoxide (DMSO) and diluted further by aCSF to a final DMSO concentration of 0.01%.

### 2.7. Statistical analysis

Analyses of variance (ANOVAs) were carried out using StatView 5.0 (SAS Institute Inc, NC) and used to determine significant main effects and interactions. Post hoc ANOVAs and Fisher's protected least significant difference comparisons, with the p-value set at 0.05, were used to further localize differences.

## 3. Results

In order to investigate the influence of various estrogen receptors in mediating the enhancement of the synaptic response, hippocampal slices were prepared from WT, ERαKO, and ERβKO mice. For a subset of slices, full input-output curves of the CA3-CA1 synaptic response amplitude were obtained prior to setting the baseline for drug studies. An examination of the input-output curves indicated no genotype (WT, n = 32; ERαKO, n = 18; and ERβKO, n = 16) difference in synaptic transmission **(Fig 1)**.

The baseline stimulation intensity was set to evoke a half-maximal amplitude synaptic response and stable EPSP responses were recorded for at least 10 min prior to drug application. The synaptic response was recorded during bath application of EB (100 pM), the GPER1 selective agonist, G1 (100 nM), the ERα-selective agonist, PPT (100 nM), or the ERβ-selective agonist, DPN (1 μM). For all genotypes and all vehicle treatments, no effect of vehicle application was observed on the synaptic response (WT: 0.3 ± 2.0%, n = 11/9, slices/animals; ERαKO: 2.6 ± 1.3%, n = 4/4, slices/animals; ERβKO: 0.8 ± 1.0%, n = 6/6, slices/animals).

The change in EPSP slope for each genotype under the various treatment conditions is illustrated in Figure 2. The percent change in the response from baseline, measured 45 min after drug application, was examined using an ANOVA for genotype and treatment. The results indicated a treatment effect [$F(3,132) = 5.7$, $p < 0.005$] in the absence of a genotype difference or an interaction of treatment and genotype. Post hoc analyses indicate that the increase in the synaptic response for EB was greater than that observed for PPT and DPN. The response to G1 was similar to that observed for EB and was increased relative to PPT and tended ($p = 0.056$) to increase relative to DPN application. It is expected that the effects of receptor selective agonists, PPT and DPN, should differ for ERαKO and ERβKO animals. Therefore, subsequent ANOVAs were conducted within each genotype. For WT animals, an ANOVA indicated a significant effect of treatment [$F(3,53) = 2.9$, $p < 0.05$] and post hoc analyses confirmed that the response to EB was greater than that for PPT and DPN (Fig 2E). The effect of G1 was intermediate and not different from the other three treatment groups. For ERαKO animals, an ANOVA indicated a significant effect of treatment [$F(3,36) = 5.2$, $p < 0.005$] and post hoc analyses indicated that the response to PPT was reduced relative to EB, G1, and DPN (Fig 2E). For ERβKO animals, an ANOVA indicated a tendency ($p = 0.067$) for a treatment effect and post hoc analyses indicated that the response to DPN was reduced relative to that for EB (Fig 2E). The results suggest that GPER1 is a major contributor to the rapid increase in synaptic strength, and ERα and ERβ provide only a modest involvement in the EB-mediated increase in synaptic responses at CA3-CA1 hippocampal synapses in female mice.

In order to further investigate the role of GPER1 in the rapid increase in synaptic transmission, we attempted to occlude the effect of EB by prior application of G1 in slices obtained from WT mice. Synaptic responses were increased 15.7 ± 6.6% (n = 4) during the 40-45 min period after bath application of G1. The last 10 min of the response to G1 (~45 min following initial G1 application) was used as a new baseline and bath application of EB (100 pM) + G1 was initiated. In this case, EB failed to induce any further increase in the

EPSP (0.4 ± 1.9%, n = 4) **(Fig 3)**. The GPER1 selective antagonist, G15 (100 nM) had no effect on the baseline synaptic response in WT mice (n = 10). In the presence of G15, EB-induced a small 5 ± 2% increase in synaptic strength, which was significantly greater than baseline, but considerably reduced relative to EB alone [$F(1,37) = 4.5$, $p < 0.05$] **(Fig 4)**. The residual increase under GPER1 blockade may have been due to activation of ERα and ERβ. Indeed, G15 (100 nM) failed to attenuate the modest potentiation in the EPSP response induced by the ERα-selective agonist, PPT (100 nM, n = 6) and the ERβ-selective agonist, DPN (1 μM, n = 6) **(Fig 4)**.

Under some conditions, the rapid increase in the synaptic response can result from an increase in transmitter release (Smejkalova and Woolley, 2010). To examine possible genotype difference in presynaptic function, paired-pulse facilitation (PPF) ratio of the synaptic response was examined during baseline recording, prior to EB application (pre EB) and compared with the PPF ratio averaged for 5 min and measured 45 min following bath application of EB (post EB). An ANOVA constructed on the PPF ratios indicated no genotype or treatment effect for WT (pre EB: 1.65 ± 0.04, post EB: 1.65 ± 0.04, n = 21 slices), ERαKO (pre EB: 1.67 ± 0.07, post EB: 1.67 ± 0.07, n = 8 slices), and ERβKO (pre EB: 1.55 ± 0.05, post EB: 1.57 ± 0.05, n = 12 slices). Studies of biochemical modifications associated with estradiol treatment suggest that the increase in the synaptic response depends on activation of ERK (Bi et al., 2001; Zadran et al., 2009). Indeed, in WT mice pre-incubation with the ERK antagonist, PD98059 (20 μM, n =7 slices), 30 min prior to EB application, completely blocked the enhancement induced by bath application of EB **(Fig 5)**. To examine ERK activation, hippocampal slices were prepared from WT, ERαKO, and ERβKO (n = 4 mice per genotype) and slices (4-5 slices per time point) were collected before and 10 and 30 min after EB treatment for Western blot (**Fig 6A**). The pERK/total ERK ratios for ERK1 and ERK2 were normalized to time 0 and an ANOVA indicated an effect of time for ERK1 [$F(2,18) = 8.69$, $p < 0.005$] and for ERK2 [$F(2,18) = 9.74$, $p < 0.005$] in the absence of a genotype difference. Post hoc test indicate that pERK1 increased at 10 min relative to the baseline and the 30 min time point and ERK2 increased at 10 relative to the baseline (**Fig 6B**).

To examine the role of GPER1 in contributing to ERK1/2 activation, hippocampal slices were prepared from WT mice; slices were exposed to G15 (100 nM) for at least 40 min and then slices were collected before (0 min, n = 8/4 slices/animals) and 10 min after EB (100 pM) application (10 min, n = 8/4, slices/animals). The pERK/total ERK ratios for ERK1 and ERK2 were normalized to time 0 and an ANOVA indicated no effect of EB treatment in the presence of the GPER1 antagonist (**Fig 6D**).

## 4. Discussion

The main finding of the current study is that GPER1 is a major component of the EB-mediated increase in ERK activation and increase in CA3-CA1 synaptic response at hippocampal synapses in female ovariectomized mice. In considering the magnitude of the response, agonists of either ERα or ERβ resulted in a modest enhancement of the synaptic response. The greater effect of EB and G1 relative to PPT and DPN indicates that differential responsiveness is due to mechanisms which are independent of classical estrogen

receptors. Previous studies have provided evidence to suggest that more than one estrogen receptor subtype contributes to the rapid EB-mediated increase in synaptic transmission in the hippocampus (Foster et al., 2008; Fugger et al., 2001; Lebesgue et al., 2010; Smejkalova and Woolley, 2010; Tanaka and Sokabe, 2013; Zadran et al., 2009); however, the relative contribution of each receptor is unclear. An issue for trying to distinguish individual estrogen receptor contributions is the selectivity of the different receptor agonists. Despite differences in affinity, some cross talk can occur, particularly at higher doses (Filardo et al., 2000; Thomas and Dong, 2006). The dose of receptor agonists employed was selected in order to minimalize interactions with the other estrogen receptors (Rossi et al., 2010; Stauffer et al., 2000). Importantly, the ERα and ERβ selective agonists did not increase the synaptic response in animals the lacked the relevant receptor, confirming selectivity for the doses employed. Interestingly, PPT and DPN produced only a small increase in the synaptic response relative to EB or the GPER1 agonist, suggesting that ERα and ERβ provide only a modest contribution to synaptic enhancement relative to GPER1. Alternatively, it is likely that receptor selective agonists mainly act on receptor subtype homodimers (Powell and Xu, 2008), resulting in the activation of a limited number of receptors. In contrast, estradiol acts on heterodimers as well as homodimers. Thus, receptor selective agonists may act on fewer receptors, which may have limited the ability to rapidly increase synaptic transmission. Regardless, the results indicate that ERα and ERβ homodimers provide a relatively modest contribution to the rapid increase in synaptic strength at CA3-CA1 synapses.

The enhanced responsiveness of EB over selective agonists may be due to the action of estradiol metabolites produced by cytochrome p450 mediated hydroxylation (Sugita et al., 1987). Hydroxylated estradiol metabolites can exert effects on neurons, which are independent of classical estrogen receptors (Philips et al., 2004; Zhu and Conney, 1998). The fact that G1 mimicked EB, and G15 blocked the EB-induced increase in synaptic transmission would suggest that metabolites could be acting on GPER1. However, while it is unknown whether estradiol metabolites bind to GPER1, the available research suggests that hydroxylated estradiol metabolites either act as antagonists or do not act through GPER1 (Chourasia et al., 2015; Duncan et al., 2012). In addition, metabolism of estradiol by hydroxysteroid dehydrogenases produces estrone (He et al., 1999; Steckelbroeck et al., 2003; Yang et al., 2007). Estrone has a lower affinity for ERα, ERβ, and GPER1 (Bhavnani, 2003; Kuiper et al., 1997; Thomas et al., 2005). Moreover, in many cases estrone influences cell physiology (Barha and Galea, 2010; Budziszewska et al., 2001; Kelly et al., 1980; Mermelstein et al., 1996; Thomas et al., 2005) and cognitive function (McClure et al., 2013) in a manner different from estradiol. Together, the results indicate that the enhanced responsiveness of EB over selective agonists is not due to EB metabolites.

Endogenous levels of estradiol may have contributed to agonist effects on synaptic transmission. Locally synthesized estradiol in the hippocampus is in the range that can preferentially bind and activate ERα (Foster, 2012; Hojo et al., 2004; Kretz et al., 2004). In this case, we might expect that basal synaptic transmission or the response to applied agonists could be influenced by local estradiol acting on ERα. Importantly, no difference was observed for the input-output curves across genotypes indicating that interactions of differentially expressed receptors and local estradiol did not influence the basal synaptic transmission. Recent work emphasizes dose by receptor interactions, such that lower doses

of estradiol act through ERα and higher doses act through ERβ to influence synaptic transmission (Smejkalova and Woolley, 2010; Tanaka and Sokabe, 2013). Indeed, biphasic dose response effects have been observed for estradiol mediated ERK activation (Kuroki et al., 2000; Wong et al., 2003; Zsarnovszky et al., 2005) and synaptic transmission (Tanaka and Sokabe, 2013). Biphasic responses to increasing levels of estradiol are observed for many systems possibly due to differences in receptor affinity or the effectiveness of signal transduction for different and possibly antagonistic receptor subtypes (Bean et al., 2014; Calabrese, 2001). Thus, we might expect that higher doses of EB may activate other mechanisms and result in a different response.

The ability of each receptor subtype to enhance synaptic transmission will depend on the level of receptor expression and proximity to the synapse, as well as the effectiveness of signal transduction. For example, ERαKO mice exhibit reduced responsiveness to application of EB and viral mediated expression of ERα in CA1 pyramidal cells of ERαKO mice increases the synaptic responsiveness to EB application (Foster et al., 2008; Fugger et al., 2001), indicating that the level of ERα expression is a factor in determining the magnitude of responsiveness. In the hippocampus, ERα and ERβ expression are mainly observed in the nucleus, cytoplasm, and dendritic processes (Clarke et al., 2000; Maggi et al., 1989; Mitra et al., 2003; Mitterling et al., 2010). However, ERα and ERβ have been localized in some dendritic spines, near synapses (Milner et al., 2005; Milner et al., 2001; Romeo et al., 2005). Nevertheless, the extent of ERα and ERβ expression across spines is unclear. Differential expression of ERα and ERβ across synaptic sites could explain changes in responsiveness observed during aging or as a result of viral mediated changes in receptor expression (Bean et al., 2014; Foster, 2012).

GPER1 is observed in the brain with high levels of expression in the hippocampus (Brailoiu et al., 2007; Hammond et al., 2011; Hazell et al., 2009). However, the exact cellular location for GPER1 expression is debated (Funakoshi et al., 2006; Matsuda et al., 2008), raising the question of whether GPER1 is a cytosolic or membrane protein. Thus, it is possible that estradiol is acting on GPER1 in the cytosol to influence signaling cascades of other estrogen receptors (Gu and Moss, 1998; Mermelstein et al., 1996). Recent work indicates that GPER1 is associated with the post-synaptic density-95 protein in dendritic spines where it could interact with other receptors; including membrane bound ERα and ERβ (Akama et al., 2013). Together, the results indicate that ERα, ERβ, and GPER1 are positioned to rapidly influence synaptic transmission and the contribution of each receptor to estrogenic effects on the synaptic response is likely to depend on the relative level of receptor expression near the synapse.

A major question in the field is whether GPER1 is an independent estrogen receptor or whether, following binding of estradiol to membrane ERα or ERβ, these receptors then interact with GPER1 in the cytosol or membrane to initiate downstream signaling (Langer et al., 2010; Levin, 2009). In the current study, the GPER1 agonist increased the synaptic response to about the same extent observed for EB and occluded the enhancement of the synaptic response mediated by subsequent EB application. Furthermore, the GPER1 antagonist, G15, inhibited synaptic enhancement and activation of ERK following EB

application. Thus, regardless, of whether GPER1 is an estrogen receptor or downstream component, it is a major factor in the EB-induced increase of the synaptic response.

The results suggest that estradiol can act through GPER1 to activate ERK, which then mediates an increase in synaptic transmission. Interestingly, several studies have observed a rapid, estrogen receptor-mediated activation of ERK followed by time-dependent decrease in kinase activity (Kuroki et al., 2000; Mannella and Brinton, 2006; Zhao and Brinton, 2007). Desensitization of GPER1 and/or feedback mechanisms for ERK activation likely underlies the transient nature of ERK activation (Foster, 2012). In contrast, the increase in synaptic strength was rapid but stable; suggesting that continued ERK activation was not required to maintain synaptic transmission. It is unclear if the enhanced transmission requires the continued activation of estrogen receptors.

In summary, our results demonstrate that ERα, ERβ, and GPER1 all participate in the rapid effects of EB on hippocampal synaptic function. Estradiol is thought to influence memory through genomic mechanisms involving neuronal health, growth, and synaptic plasticity (Aenlle and Foster, 2010; Bean et al., 2014; Han et al., 2013). In addition, rapid effects of estradiol on ERK activation and neuronal physiology may modulate memory (Bean et al., 2014; Boulware et al., 2013). The current study suggests that if rapid signaling and enhanced synaptic transmission contribute to learning and memory, then activation of any single estrogen receptor subtype could improve memory; however, the contribution of each receptor will depend on the expression/function and localization of receptors.

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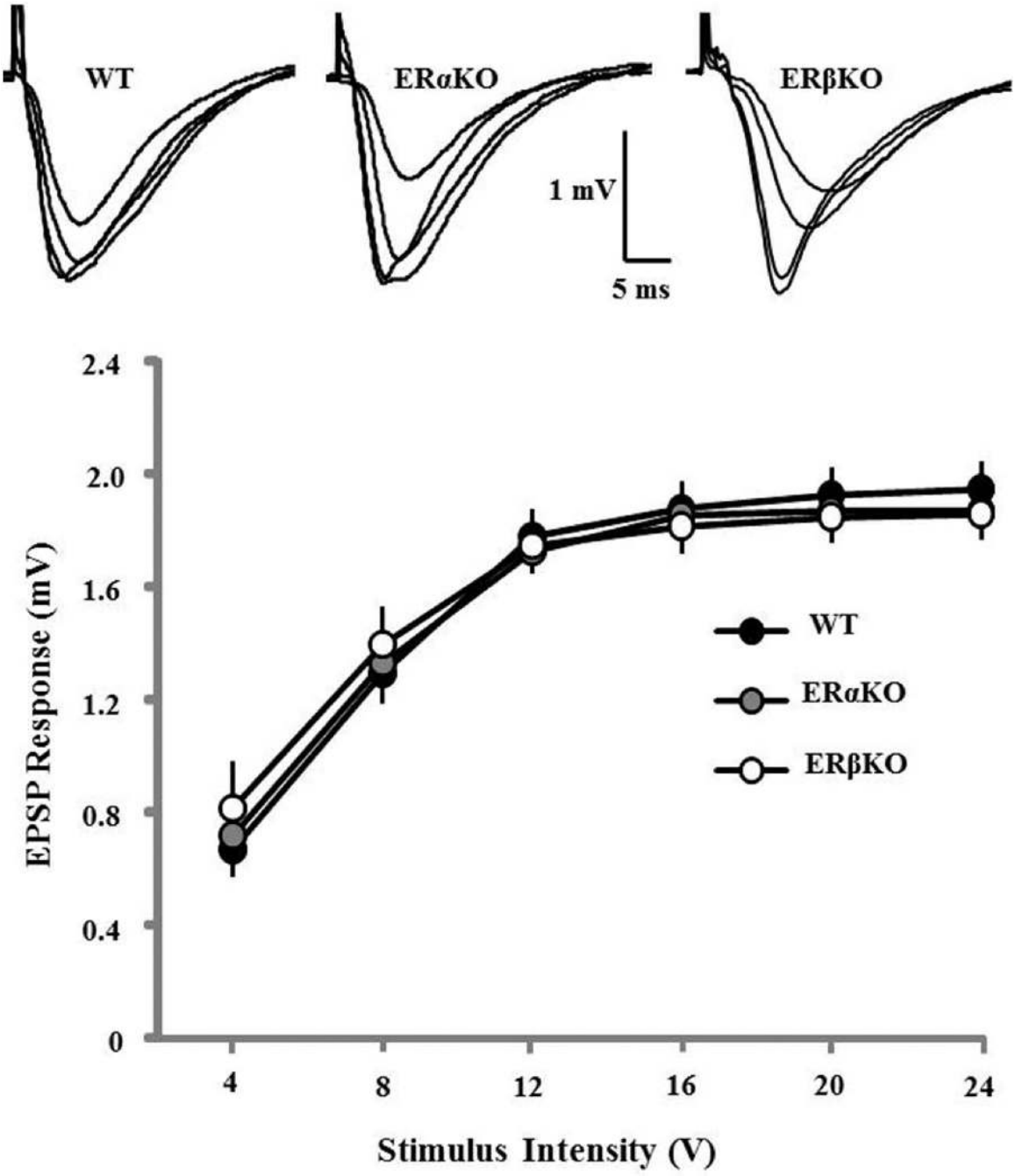


**Figure 1.**

No difference in baseline synaptic transmission across WT, ERαKO, and ERβKO mice. Input–output curves of mean baseline EPSP response vs. stimulus intensity for WT (filled circle, n = 32 slices), ERαKO (gray circle, n = 18 slices), and ERβKO (open circle, n = 16 slices) mice. Individual EPSP traces from WT, ERαKO, and ERβKO mice obtained at 4, 8, 16, and 24 volts stimulus intensity.

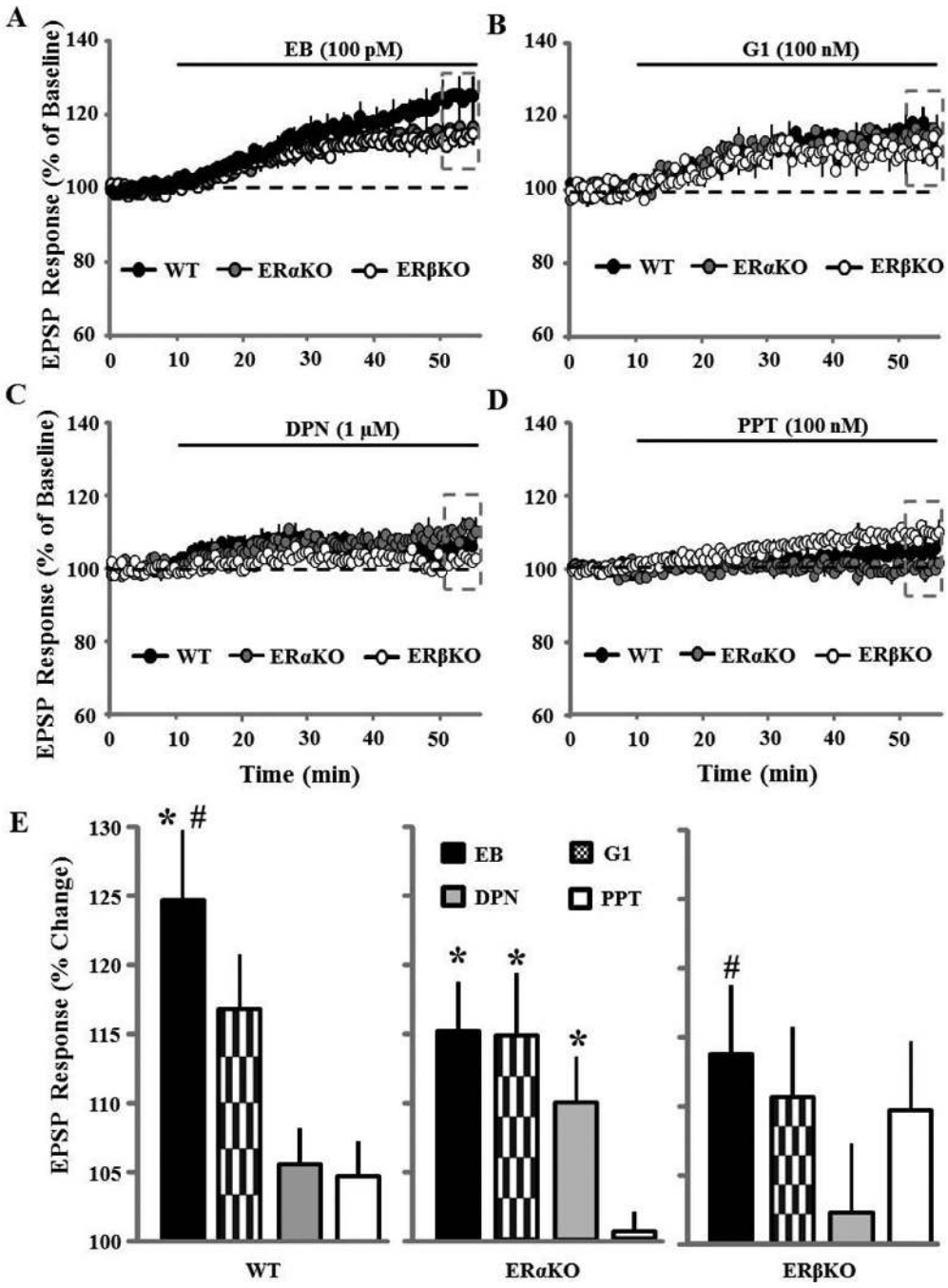


**Figure 2.**

Effect of estrogen receptor selective agonists on synaptic transmission in hippocampal slices obtained from WT, ERαKO, and ERβKO mice. Time course of the field EPSP measurements for WT (filled circle), ERαKO (gray circle), and ERβKO (open circle) mice obtained 10 min before and 45 min after application of **A)** EB (100 pM, WT: n = 29 slices; ERαKO: n = 11 slices; and ERβKO: n = 14 slices) mice, **B)** G1 (100 nM, WT: n = 11 slices; ERαKO: n = 6 slices; and ERβKO: n = 10 slices), **C)** DPN (1 uM, WT: n = 8 slices; ERαKO: n = 11 slices; and ERβKO: n = 11 slices), or **D)** PPT (100 nM, WT: n = 9 slices;

ERαKO: n = 12 slices; and ERβKO: n = 12 slices). **E)** Summary diagram showing the increase in the synaptic response relative to baseline and measured 45 min after drug application for EB (filled bars), G1 (checkered bars), DPN (gray bars), and PPT (open bars) in slices obtained from WT, ERαKO, and ERβKO mice. The dashed line box in **A-D** indicates the 40-45 min post drug application. Asterisk indicates an increase in the synaptic response relative to PPT. Pound sign indicates an increase in the synaptic response relative to DPN.

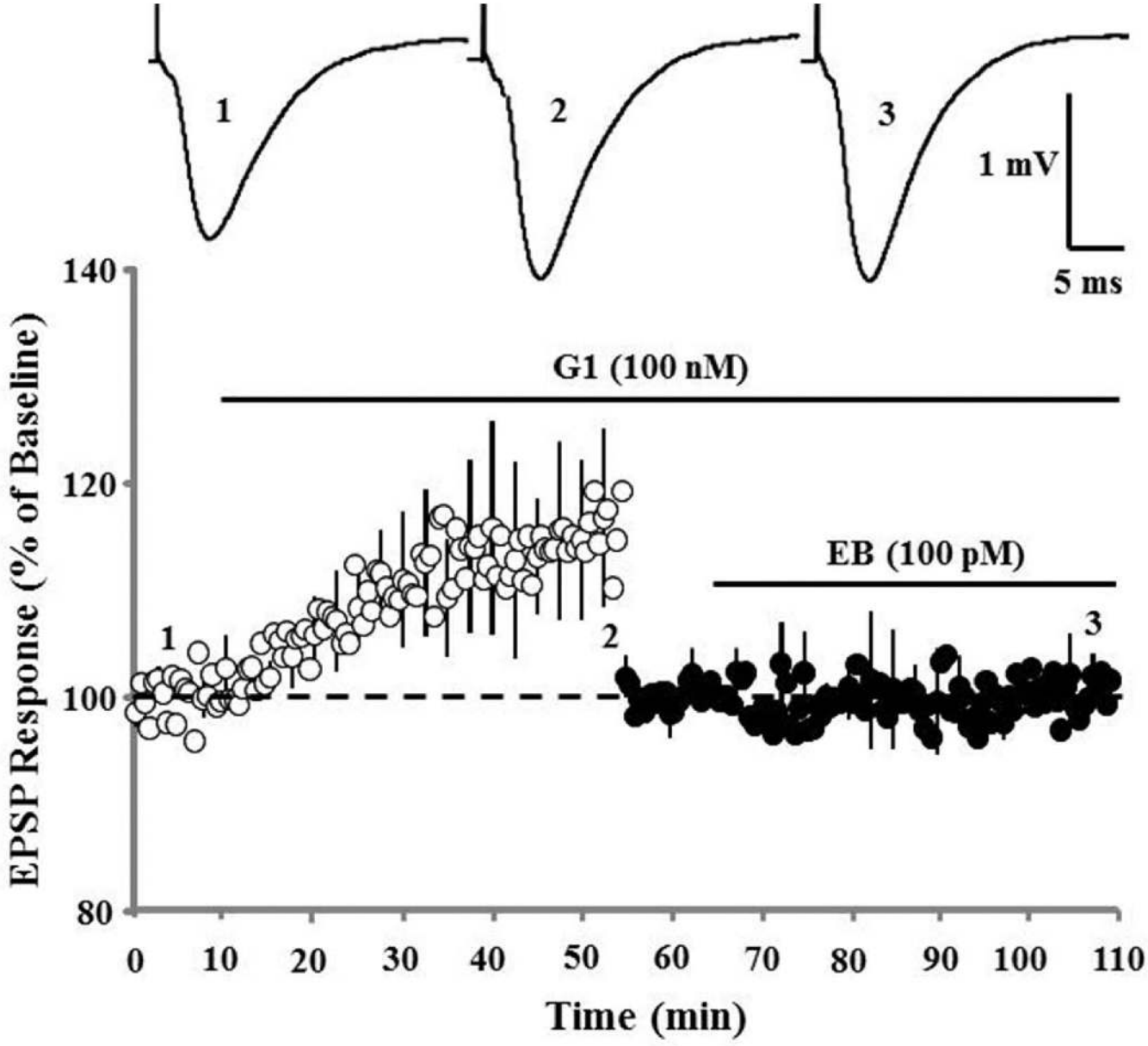


**Figure 3.**

GPER1 selective agonist, G1 occluded the EB-induced enhanced synaptic responses in hippocampal slices obtained from WT animals. The time course of field EPSP measurements obtained from hippocampal slices 10 min before and 45 min after bath application of G1 (100 nM, open circles, n = 4 slices). Baseline was re-normalized 45-60 min following the start of G1 application (filled circles) and EB was bath applied in presence of G1. EPSP traces (average of 5 continuous sweeps) from the same slice obtained under control (1), G1 (2), and G1+EB (3) conditions.

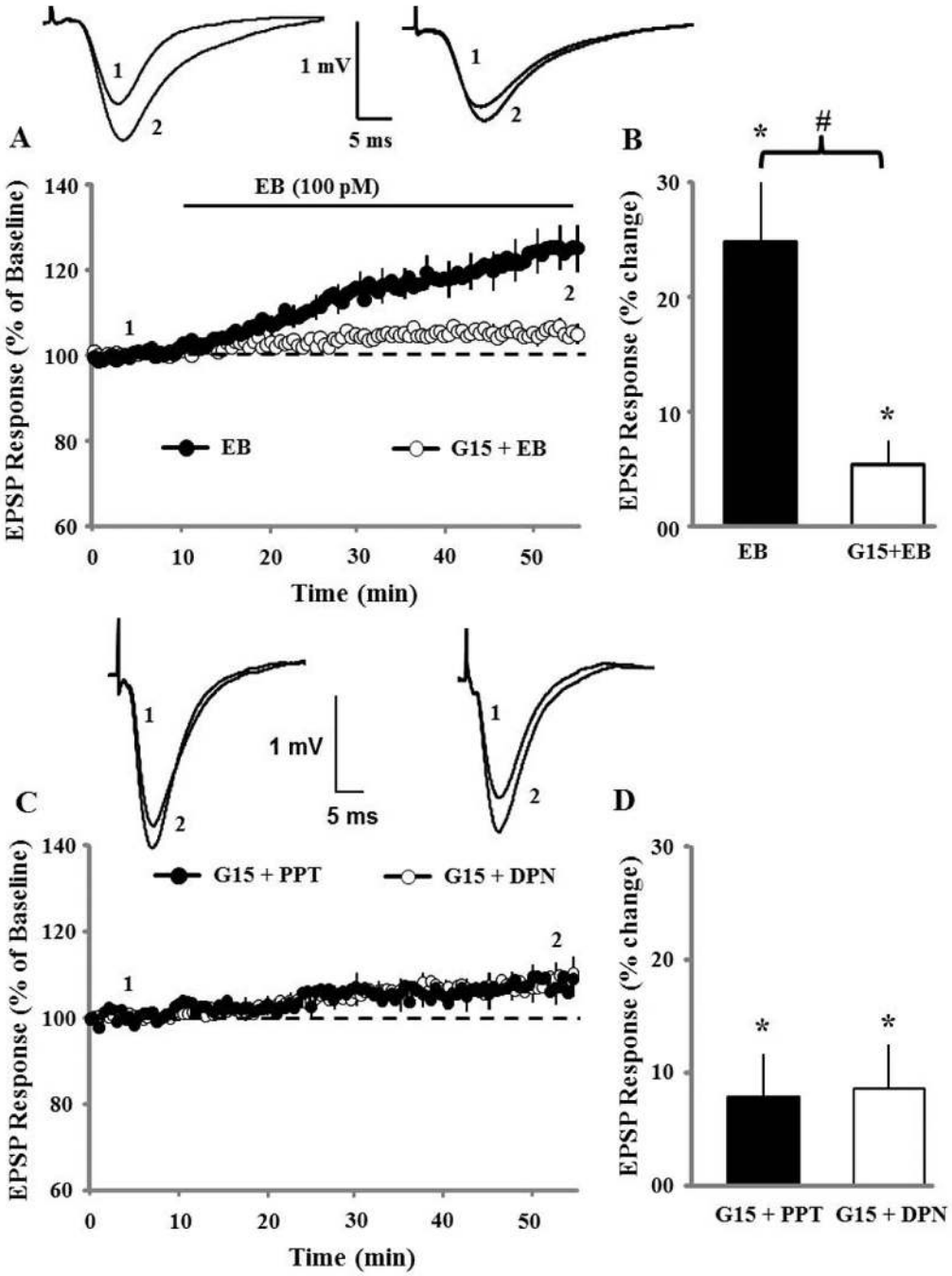


**Figure 4.**

The GPER1 antagonist, G15, attenuated the EB-induced increase of the synaptic response in hippocampal slices obtained from WT animals. **A)** Time course of the field EPSP measurements obtained from hippocampal slices 10 min before and 45 min after EB application in absence (filled circle, n = 29 slices) and presence of G15 (100 nM, open circle, n = 10 slices). The EPSP traces (average of 5 continuous sweeps) at the time points (1 and 2) indicated in the time course for bath application of EB alone (left) and EB in presence of G15 (right). **B)** Bar diagram showing the percentage change in the magnitude of EPSP

during 40-45 min following bath application of EB in absence (EB, filled bar) and presence of G15 (open bar). **C)** Time course of the field EPSP measurements obtained from hippocampal slices 10 min before and 45 min bath application of PPT (filled circle, n = 6 slices) and DPN (open circle, n = 6 slices) in presence of G15 (100 nM). **D)** Bar diagram showing the percentage change in the magnitude of EPSP during 40-45 min following bath application of PPT (filled) and DPN (open) in presence of G15. The EPSP traces at the time points (1 and 2) indicated in the time course for bath application of PPT (left) and DPN (right) in presence of G15. Asterisk indicates significant difference from baseline (dotted line) and pound sign indicates significant treatment effect.

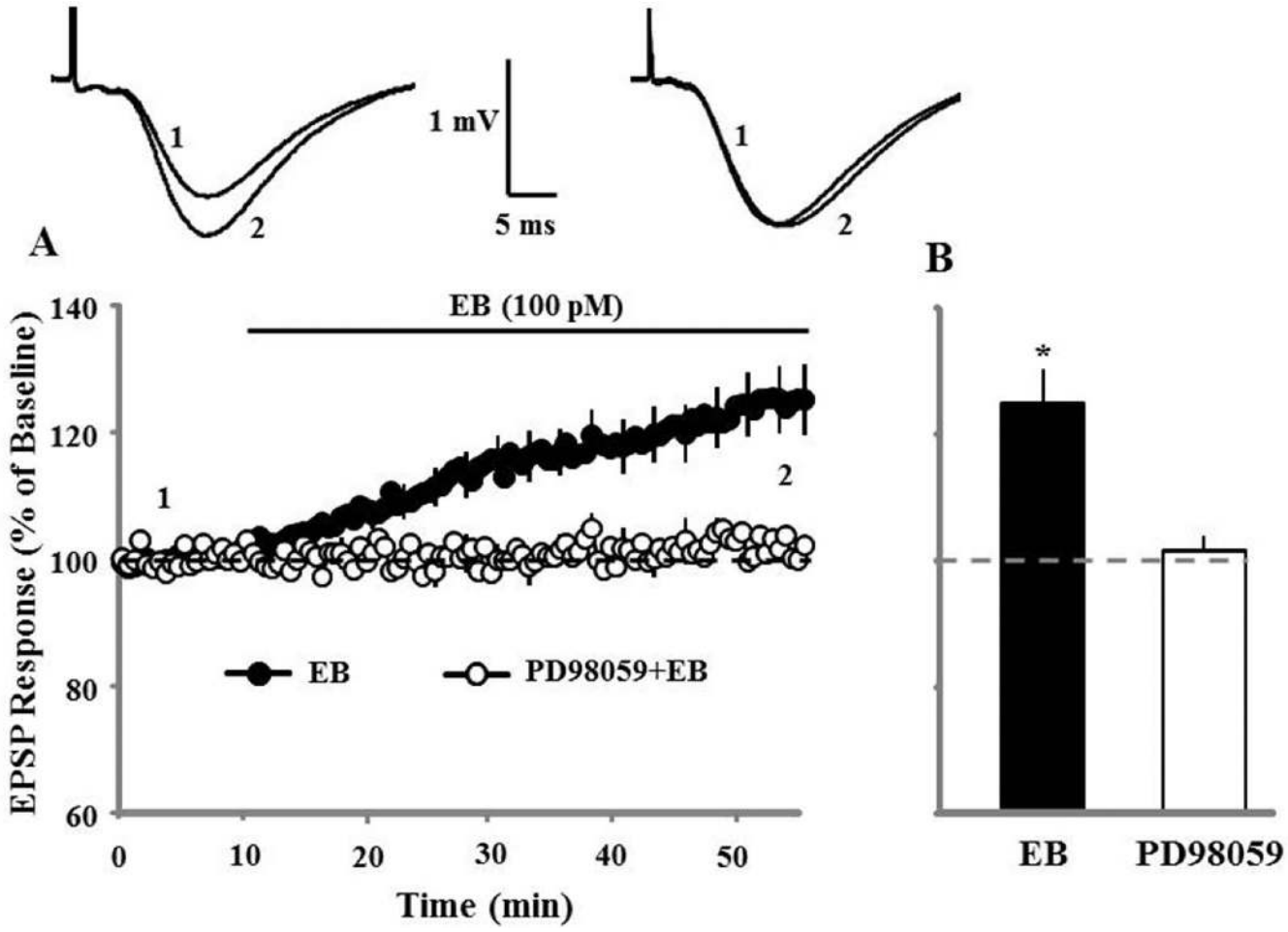


**Figure 5.**

ERK antagonist, PD98059, prevented the EB-induced enhancement of synaptic responses in hippocampal slices obtained from WT animals. **A)** Time course of the field EPSP measurements obtained from hippocampal slices 10 min before and 45 min after EB (100 pM) application in absence (filled circle, n = 29 slices) and presence of PD98059 (20 μM , open circle, n = 7 slices). **B)** Bar diagram showing the average magnitude of EPSP following bath application of EB in absence (EB, filled bar) and presence of PD98059 (open bar). Star indicates significant difference from baseline (dotted line). EPSP traces (average of 5 continuous sweeps) at the time points (1 and 2) indicated in the time course for bath application of EB alone (left) and EB in presence of PD98059 (right).

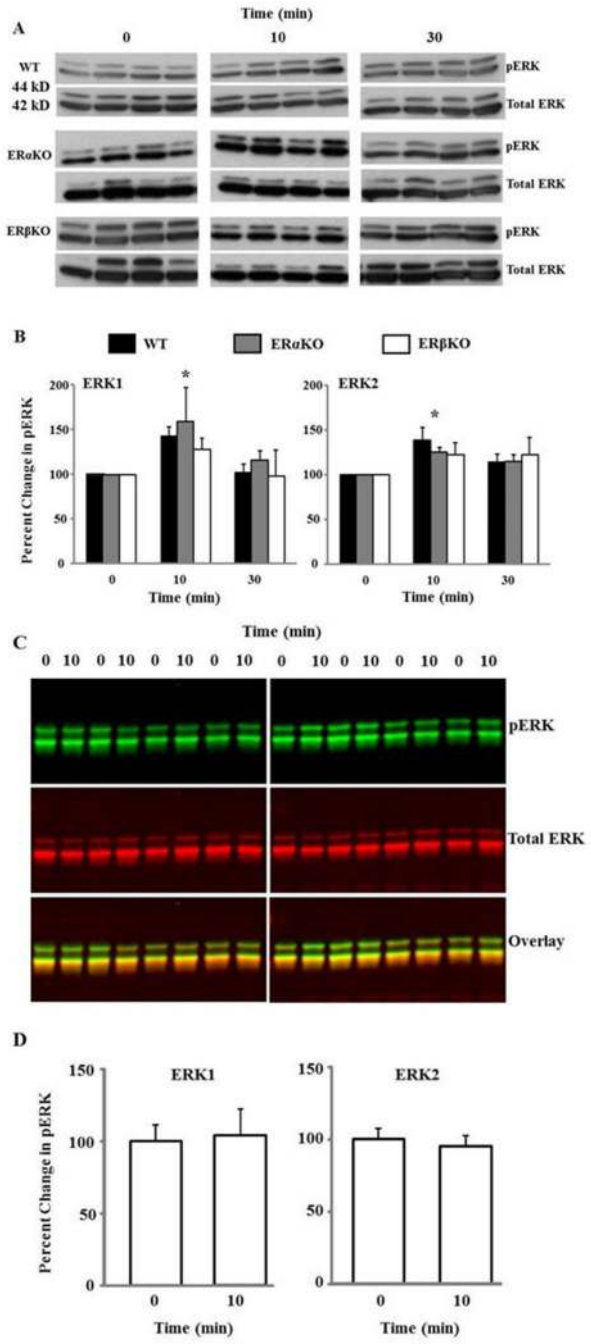


**Figure 6.**

EB-induced ERK activation in hippocampal slices. **A)** For each animal (n = 4 per genotype), hippocampal slices (4-5) were collected at times 0, 10, and 30 min after application of EB (100 pM). Western blots of hippocampal slice lysates for phosphorylated (pERK) and total ERK1 (44 kD) and ERK2 (42 kD) from the different genotypes (WT, ERαKO, and ERβKO). **B)** The bars indicate the mean (+SEM) pERK/total ERK ratios for ERK1 and ERK2 for WT (black), ERαKO (gray), and ERβKO (open), normalized to the 0 time point. **C**) Western blots of hippocampal slice lysates for phosphorylated (pERK, green), total ERK

(red), and the merged image (lower panel) from the hippocampal slices exposed to G15 (100 nM) for at least 40 min and collected at times 0 or 10 min following application of EB (100 pM, n = 8/4, slices/animal at each time point). **D)** The bars indicate the mean (+SEM) pERK/total ERK ratio for ERK1 and ERK2 normalized to the 0 time point. The asterisks in **B** indicate a significant ($p < 0.05$) increase in the pERK/total ERK ratio across genotype relative to the baseline.