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Activation of estrogen receptor-β regulates hippocampal synaptic plasticity and improves memory

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Estrogens have long been implicated in influencing cognitive processes, yet the molecular mechanisms underlying these effects and the roles of the estrogen receptors alpha (ERα) and beta (ERβ) remain unclear. Using pharmacological, biochemical and behavioral techniques, we demonstrate that the effects of estrogen on hippocampal synaptic plasticity and memory are mediated through ERβ. Selective ERβ agonists increased key synaptic proteins *in vivo*, including PSD-95, synaptophysin and the AMPA-receptor subunit GluR1. These effects were absent in ERβ knockout mice. In hippocampal slices, ERβ activation enhanced long-term potentiation, an effect that was absent in slices from ERβ knockout mice. ERβ activation induced morphological changes in hippocampal neurons *in vivo*, including increased dendritic branching and increased density of mushroom-type spines. An ERβ agonist, but not an ERα agonist, also improved performance in hippocampus-dependent memory tasks. Our data suggest that activation of ERβ can regulate hippocampal synaptic plasticity and improve hippocampus-dependent cognition.

There are two well characterized estrogen receptors, ERα and ERβ, described in mammals[1,2]. They both function as nuclear transcription factors modulating expression of their target genes in response to changes in estrogen levels. ERα and ERβ share similar domain organization and show substantial sequence homology but have differences in their binding affinities for ligands[3]. The two receptors are not functionally interchangeable, as demonstrated by studies with ERα (*Esr1*$^{-/-}$) and ERβ (*Esr2*$^{-/-}$) knockout mice. For example, *Esr1*$^{-/-}$ mice manifest severely impaired reproductive function leading to infertility[4], whereas *Esr2*$^{-/-}$ mice retain fertility[5], suggesting different roles for these two receptors in estrogen biology.

Supporting this functional divergence, ERα and ERβ have been shown to be differentially expressed throughout the rodent brain[6]. ERα and ERβ are both expressed in the hippocampus, a region of the brain involved in cognitive function[7]. Within the hippocampus, ERβ is expressed in the CA3 stratum lucidum, CA1 stratum radiatum[8] and dentate gyrus regions[9] and at the subcellular level is found in axons, dendrites and dendritic spines[10].

The importance of estrogen in cognitive function has been highlighted by examining cognition in relation to phases of the menstrual cycle, menopausal symptoms, circulating hormone levels and aging[11–13]. In addition 17-β-estradiol (hereinafter referred to as estradiol), a main form of estrogen, has been reported to enhance learning in a range of rodent memory tasks, including the Morris water maze and inhibitory avoidance tasks[14,15]. Several mechanisms have been identified through which estrogen may influence cognitive activity. Estradiol has been shown to promote the formation of new dendritic spines and excitatory synapses in the hippocampus[16,17], increase the expression of NMDA receptor (NMDAR) subunit NR2B (ref. 18) and enhance NMDAR-mediated synaptic activity and long-term potentiation (LTP)[19,20]. Estradiol has also been shown to increase cyclic AMP response element–binding protein (CREB) phosphorylation[21], a process which has been strongly implicated in the formation of long-term memory.

The identity of the estrogen receptor responsible for mediating the effects of estrogen on cognition has not yet been fully resolved. However, accumulating evidence supports a key role for ERβ. For example, when compared to similarly treated wild-type mice, *Esr2*$^{-/-}$ mice treated with estradiol show impairments in acquisition of a spatial reference memory, implicating a role for ERβ in hippocampus-dependent cognition[22]. In support of this, we have previously shown that *Esr2*$^{-/-}$ mice not only show profound memory impairment in the hippocampus-dependent contextual fear conditioning task but also show deficits in hippocampal neuronal activity, both in terms of their synaptic input-output curves and their expression of LTP in the CA1 region of the hippocampus[23]. It has also been shown that the ERβ

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selective agonist 2,3-bis(4-hydroxyphenyl)-propionitrile, but not the ERα selective agonist 4,4′,4′′-[4-propyl-(1*H*)-pyrazole-1,3,5-triyl]trisphenol (PPT), enhances spatial memory in ovariectomized (OVX) rats in a Morris water maze task[24].

We therefore investigated whether activation of ERβ is sufficient to drive the molecular events (for example, CREB phosphorylation and glutamatergic excitatory synapse modifications) that promote synaptic plasticity (for example, spine morphology and LTP) leading to the behavioral end points of learning and memory enhancement. Using specific pharmacological and genetic tools, we have been able to show that ERβ activation is capable of improving performance in memory tasks. The accompanying ERβ-induced changes in expression of key synaptic proteins, synaptic structure and activity-dependent plasticity are highly plausible routes to this enhanced cognitive function.

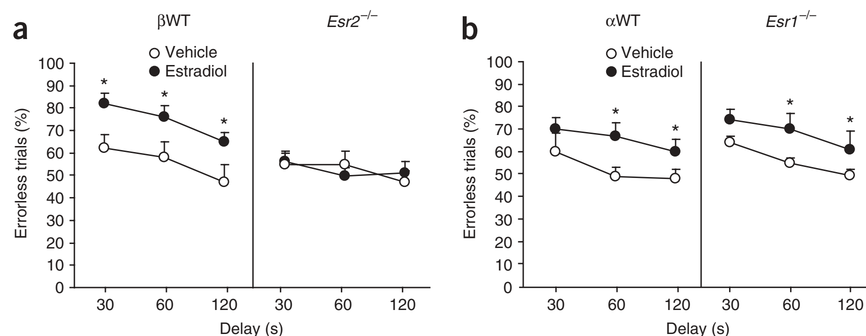

Figure 1 Hippocampus-dependent spatial memory is enhanced by estradiol in OVX WT and *Esr1*−/− mice but not in OVX *Esr2*−/− mice. (**a**,**b**) Percentage of errorless trials by mice in a delayed nonmatching-to-sample procedure showed a significant increase in βWT but not *Esr2*−/− mice (**a**) and an increase in both αWT and *Esr1*−/− (**b**) (*$P < 0.05$ compared to vehicle; $n = 20$). Data presented as mean of percentage of errorless trials ± s.e.m.

RESULTS

Estradiol improves cognition in an ERβ-dependent manner

Estradiol has been implicated in producing cognition-enhancing effects, but the estrogen receptor responsible for mediating these effects is still not clearly defined. We examined the relative roles of ERα and ERβ on estradiol-mediated cognitive enhancement in a hippocampus-dependent Y-maze task using knockout and wild-type OVX mice.

Estradiol treatment (5 μg, subcutaneously (s.c.)) of wild-type littermates of *Esr2*−/− mice (βWT) mice significantly increased the percentage of errorless trials at delays of 30, 60 and 120 s, compared to vehicle treatment (**Fig. 1a**). These effects were absent in the *Esr2*−/− mice (**Fig. 1a**). In contrast, estradiol treatment in the *Esr1*−/− mice significantly increased the percentage of errorless trials made at 60 and 120 s delays, compared to vehicle treatment. This effect was similar to the effects seen in the wild-type littermates of *Esr1*−/− mice (αWT) mice (**Fig. 1b**). These results suggest that ERβ mediates the effects of estradiol on enhanced memory retention in a hippocampus-dependent task.

ERβ agonists increase CREB phosphorylation *in vivo*

To understand the molecular events driven by ERβ in the hippocampus we used a number of specific ERα and ERβ agonists (**Table 1**). We first examined the effects of these compounds on the distribution of estrogen receptors, in particular on the levels accumulated in the nuclei of hippocampal cells using an *in vitro* nuclear exchange assay. Hippocampal extracts from animals treated with either ERβ selective agonists, WAY-200070 or WAY-202779 (10 mg per kilogram body weight, s.c.) showed a significant increase in [^{3}H]estradiol binding compared to the vehicle-treated control group (**Fig. 2a**). Similar results were observed with estradiol (5 μg, s.c.) and an ERα-specific agonist, PPT (10 mg kg^{-1}, s.c.) (**Fig. 2a**). These results indicate that all estrogen receptor ligands, after systemic dosing, can pass through the blood-brain barrier, bind to their receptor and induce receptor translocation into the nucleus as measured by the elevation in [^{3}H]estradiol binding.

Estrogens have also been shown to exert acute effects on synaptic physiology involving the activation of signaling cascades such as the protein kinase A and mitogen-activated protein kinase (MAPK) pathways. These effects occur too rapidly for the classical genomic littermates of *Esr1*−/− mice effects of estrogen[21,25]. To assess the nongenomic signaling potential of estrogen ligands, we monitored changes in the abundance of pCREB, which has been shown to be modulated as a result of estradiol treatment[21]. In OVX rats, dosing with either ERβ selective agonist (WAY-200070 or WAY-202779; 10 mg kg^{-1}, s.c.) or estradiol (5 μg, s.c.) induced significant increases in pCREB levels within 15 min (**Fig. 2b**). In contrast, there was no significant increase in pCREB in OVX rats dosed with the ERα agonist PPT (**Fig. 2b**).

Because of the superior brain penetration of WAY-200070 as compared to WAY-202779 (**Table 1**), we characterized the effects of WAY-200070 in further detail. Analysis of a time window of 4 h after dosing revealed that WAY-200070 significantly increased both the levels of estrogen receptors in the nucleus (**Fig. 2c**) and pCREB (**Fig. 2d**) at 15 and 60 min time points. These effects returned to baseline levels by 240 min (**Fig. 2c,d**). To confirm that the effects of WAY-200070 were specifically mediated by ERβ receptors, we used *Esr2*−/− mice. βWT OVX female mice dosed with WAY-200070 (10 mg kg^{-1}, s.c.) showed significant increases in both estrogen receptor levels in the nucleus, as measured by [^{3}H]estradiol binding (**Fig. 2e**), and pCREB levels as compared to vehicle-dosed animals (**Fig. 2f**). In

Table 1 Binding affinity (IC_{50}), pharmacokinetics and blood-brain barrier penetration of WAY-200070, WAY-202779 and PPT

	IC_{50}[a] (nM)		T_{max}[b,c] (h)		C_{max}[b,c] (ng ml^{-1})		AUC_{last}[b,c] (ng h ml^{-1})		
Compound	ERα	ERβ	Plasma	Brain	Plasma	Brain	Plasma	Brain	Brain/plasma[b,c] (%)
WAY-200070	155	2.3	0.25	0.5	446	560	809	565	70
WAY-202779	227	1.8	0.25	0.5	800	246	896	262	30
PPT	0.12	49	Ref. 50	Ref. 50	Ref. 50	Ref. 50	Ref. 50	Ref. 50	Ref. 50

[a]Competitive radiometric binding assays were done with purified full-length human ERα and ERβ (ref. 50). [b]WAY-200070 and WAY-202779 were injected to C57/BL6 mice (s.c.), plasma and brain samples were collected after 15, 30, 60 and 120 min. Pharmacokinetics were determined. [c]C_{max}, maximum plasma concentration; T_{max}, time point at C_{max}; AUC_{last}, area under the curve up to the last time point.

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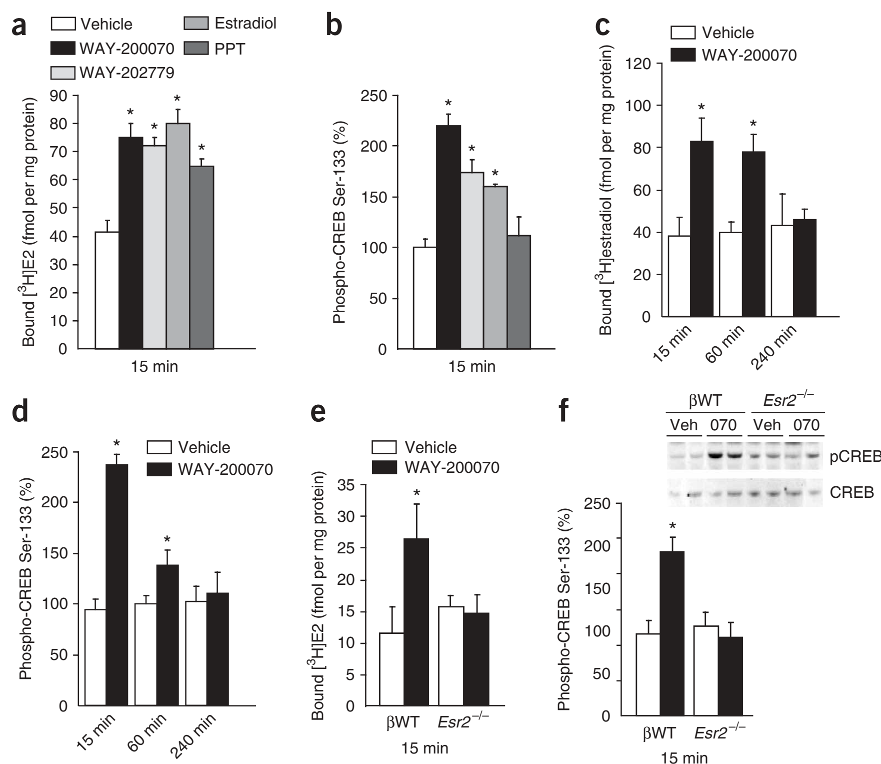

Figure 2 ERβ activation induces estrogen receptor translocation to the nucleus and increases pCREB level in rats and βWT mice, but not in *Esr2*$^{-/-}$ mice. (**a**–**d**) OVX rats ($n = 12$) or (**e**–**f**) mice ($n = 9$) were injected with either vehicle, estradiol, WAY-200070, WAY-202779 or PPT. Hippocampi were collected at indicated times. For [^{3}H]estradiol binding (**a**,**c**,**e**), data are presented as mean ± s.e.m. (*$P < 0.05$ versus vehicle; $n = 6$–9). pCREB levels (**b**,**d**,**f**) were normalized to CREB and presented as mean percentage ± s.e.m. compared to the vehicle (Veh) control (*$P < 0.05$ versus vehicle; $n = 6$–9).

contrast, the effects of WAY-200070 were absent in female *Esr2*$^{-/-}$ OVX mice (**Fig. 2e**,**f**). We observed similar results in male rats and mice (**Supplementary Fig. 1** online). Taken together, these data show that the observed effects of WAY-200070 are ERβ dependent and that the activation of ERβ, rather than ERα, modulates pCREB levels *in vivo*.

ERβ activation increases levels of synaptic proteins

Regulation of AMPA receptors (AMPARs), either by trafficking to the synapse or by phosphorylation, are critical postsynaptic modifications that regulate synaptic strength[26]. PSD-95 is a core postsynaptic scaffold protein found in most mature excitatory glutamatergic synapses. It has been shown to have a critical role at the synapse, including the regulation of AMPAR trafficking to synapses[27,28]. We evaluated the protein expression of the AMPAR subunit GluR1 and PSD-95, in addition to the presynaptic marker synaptophysin, in response to pharmacological ERβ activation.

We dosed OVX rats with either estradiol (5 μg, s.c.), the ERβ agonists WAY-200070 or WAY-202779 (10 mg kg^{-1}, s.c.), or the ERα agonist PPT (10 mg kg^{-1}, s.c.). Four hours later, we isolated hippocampi and prepared extracts. Analysis of the extracts with specific antisera showed significant increases in GluR1 and PSD-95 proteins for estradiol- and ERβ agonist–treated rats compared to vehicle treated controls (**Fig. 3**). The change in expression of PSD-95 and GluR1 seems to be ERβ mediated, as it was not observed in OVX rats treated with the ERα-specific agonist PPT or in *Esr2*$^{-/-}$ mice treated with WAY-200070 (**Fig. 3a**,**c**). No significant change was seen in any group in synaptophysin expression levels (**Fig. 3b**). In previous reports, estradiol has been shown to increase the expression of the NMDAR subunit NR2B in

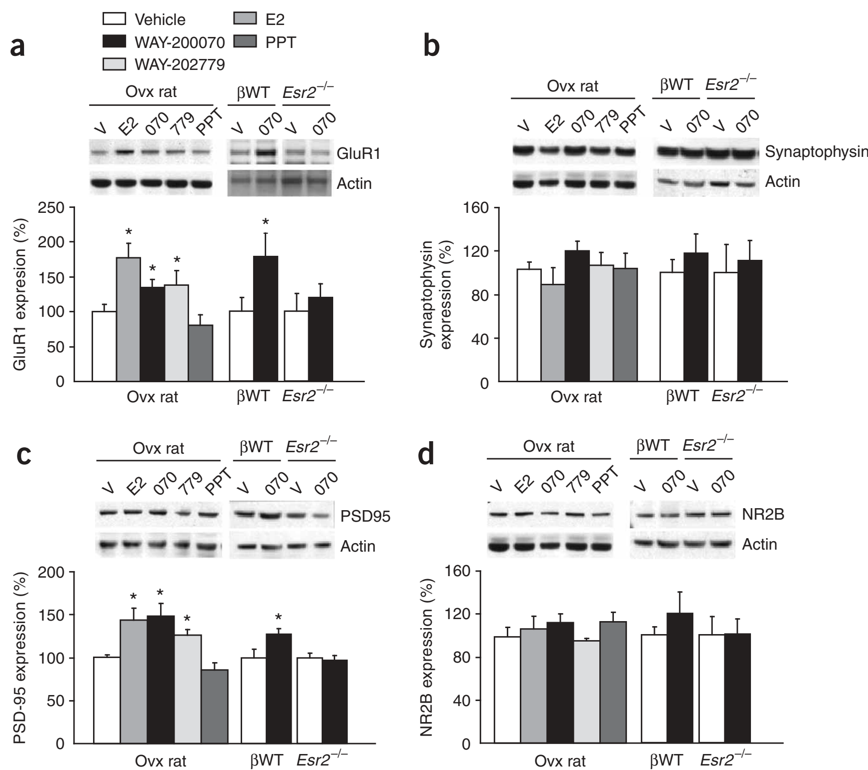

Figure 3 ERβ activation increases AMPAR GluR1 and PSD-95 expression levels in rat and mouse hippocampus. OVX rats or OVX βWT and *Esr2*$^{-/-}$ mice were injected with either vehicle (V), estradiol (E2), WAY-200070 (070), WAY-202779 (779) or PPT. Immunoblot of hippocampal extracts with antibodies to (**a**) GluR1, (**b**) synaptophysin, (**c**) PSD-95 and (**d**)NR2B. Densitometric analysis of immunoreactivity for each protein is graphed below the blot in each panel. Expression levels were normalized to actin and presented as mean percentage ± s.e.m. compared to the vehicle control (*$P < 0.05$ versus vehicle; $n = 9$–12).

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the hippocampus[18]. In our system, no significant changes were observed on NR2B expression levels when vehicle and treatment groups were compared (**Fig. 3d**). In addition, we monitored the levels of NR1, a component of all NMDARs, and saw no changes in any treatment group (data not shown). To examine whether the changes were restricted to OVX females, we conducted comparable experiments in males and obtained similar results (**Supplementary Fig. 2** online).

To further characterize which regions of the hippocampus are responsive to ERβ activation, we used quantitative immunohistochemistry in WT male mice (**Fig. 4**). WAY-200070 treatment significantly increased GluR1 immunostaining within the CA1 radial and lacunosum molecular layers, the CA3 oriens and the dentate gyrus molecular layer (**Fig. 4a,g**). The effects of WAY-200070 on GluR1 levels were absent in *Esr2*$^{-/-}$ mice (**Fig. 4f**). Notably, *Esr2*$^{-/-}$ mice had less GluR1 than wild-type mice in CA1 oriens, CA1 pyramidal and CA1 lacunosum molecular layers (**Fig. 4e**). This decrease may be a molecular explanation for the deficits in cognition and neuronal activity observed in the *Esr2*$^{-/-}$ mice[23]. There was also an increase in the presynaptic marker synaptophysin within the pyramidal and radial layers of the CA1 region as well as in the granule cells of the dentate gyrus (**Fig. 4b,h**). Although we detected an increase in PSD-95 by quantitative western blotting in total hippocampal extracts with WAY-200070 treatment, no specific region showed significantly increased expression by immunohistochemistry (**Fig. 4c**). No changes were detected in the NMDAR subunit NR1 (**Fig. 4d**). These data suggest ERβ activation can lead to specific regional changes in protein expression of GluR1 and synaptophysin.

ERβ activation regulates GluR1 trafficking and phosphorylation

It is well established that an increase in synaptic GluR1-containing AMPARs is a key contributory mechanism to the potentiation of synaptic strength seen in LTP, which itself is thought to be a cellular correlate of learning and memory[7,26,29,30]. To investigate whether the ERβ-driven increases in the pool of GluR1 was reflected in receptors being inserted into the surface membrane, we used the surface-receptor labeling technique of biotinylation in acutely prepared OVX rat hippocampal slices. We first confirmed that ERβ activation results in an increase in total GluR1 in slices. WAY-200070 (10 nM, 1 h) increased

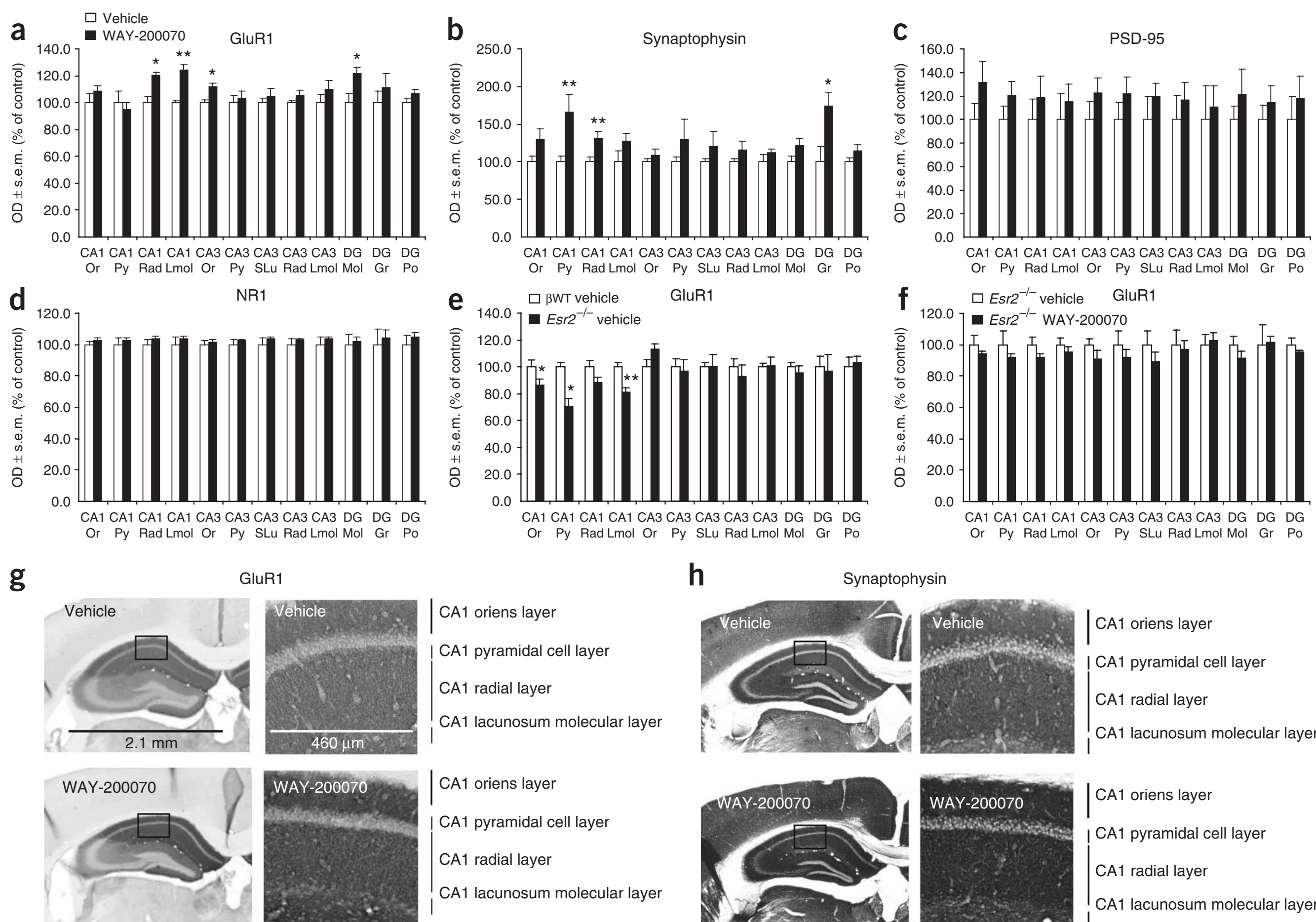

Figure 4 ERβ activation increases GluR1 and synaptophysin expression in CA1 and dentate gyrus regions. (**a**–**d**) Quantitation of protein expression from coronal sections of brains from male wild-type mice injected with WAY-200070 (10 mg kg^{-1}, s.c.) 4 h before death and immunostained for GluR1 (**a**), synaptophysin (**b**), PSD-95 (**c**) and NR1 (**d**). (**e**) Results for brains from βWT and *Esr2*$^{-/-}$ mice treated with vehicle and immunostained for GluR1. (**f**) Effect of WAY-200070 compared to vehicle treatment in *Esr2*$^{-/-}$ mice. The staining intensities were normalized to those of vehicle-treated mice and presented as the percentages mean optical densities (OD) ± s.e.m. (*$P < 0.05$ and **$P < 0.01$ versus vehicle; $n = 40$ (five mice, eight slices per mouse)). (**g**,**h**) Representative images of GluR1 (**g**) and synaptophysin (**h**) staining after vehicle or WAY-200070 treatment in wild-type mice. Rectangle in left panel indicates area magnified in right panel. Or, oriens; Py, pyramidal cell; Rad, radial; Lmol, lacunosum molecular; SLu, stratum lucidum; Mol, molecular; Gr, granule cell; Po, polymorphic.

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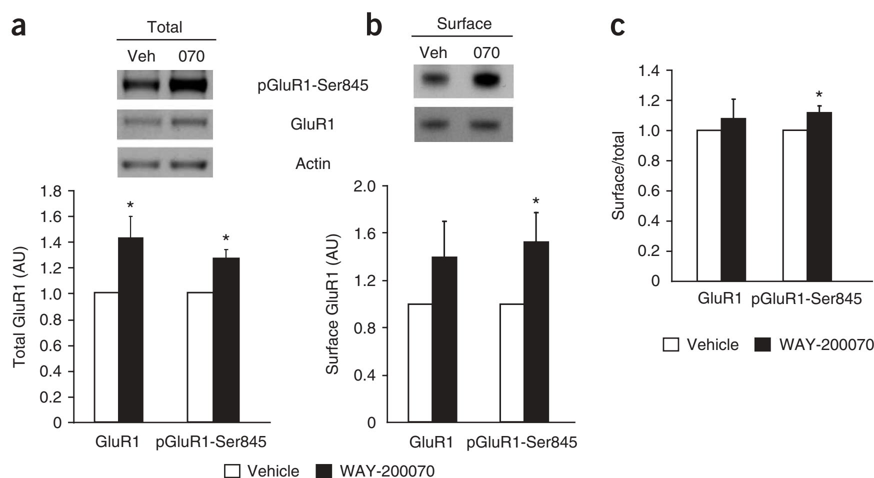

Figure 5 ERβ activation increases the surface expression of GluR1 and phosphorylation at residue Ser845. (**a**,**b**) Quantitative immunoblot analysis of OVX rat hippocampal slices treated with WAY-200070 or vehicle (Veh), followed by biotinylation. Top, immunoblots of both total and biotinylated (surface) pools of receptor, using antibodies to GluR1 and to Ser845-phospho-GluR1. Bottom, densitometric analyses of total immunoreactivity (**a**) and surface immunoreactivity (**b**) for either GluR1 or Ser845-pGluR1 proteins. Total GluR1 expression was normalized to actin and surface GluR1 immunoreactivity was normalized to total. Data are compared to vehicle treatment and presented as the mean of arbitrary units (AU) ± s.e.m. (*$P < 0.05$ versus vehicle; $n = 7$). (**c**) Ratio of surface to total.

total GluR1 levels by ~40% compared to vehicle treatment (**Fig. 5a**). The increase in GluR1 protein following ERβ activation was therefore observed in both acute slices (**Fig. 5a**) and in hippocampus from animals dosed with ERβ agonists (**Figs. 3a** and **4a**). Using surface-receptor biotinylation, we also saw a similar 40% increase in surface GluR1 in slices (**Fig. 5b**). The ratio of surface to total GluR1 after ERβ activation (1.07 ± 0.13) (**Fig. 5c**) indicates that the additional pool of total GluR1 is being proportionally inserted into the cell surface.

Phosphorylation of GluR1 on Ser845 by protein kinase A has been shown to increase AMPAR cell-surface expression[31]. Treatment of hippocampal slices with WAY-200070 increased total GluR1 Ser845 phosphorylation by approximately 30% compared to vehicle treatment (**Fig. 5a**). In parallel, we measured surface GluR1 phosphorylated on Ser845 and saw a 40% increase after ERβ activation (**Fig. 5b**). The ratio of surface to total phosphorylated GluR1 (1.12 ± 0.04) (**Fig. 5c**) indicates that phosphorylation on Ser845 promotes GluR1 trafficking to the surface, in agreement with previous studies[31]. These data suggest that ERβ activation results in increased surface GluR1 expression that, if synaptic in nature, would lead to increases in synaptic strength.

ERβ activation enhances LTP in hippocampal slices

LTP is a neurophysiological phenomenon widely thought to represent at least some of the processes that underlie learning and memory[7,29]. In order to assess the neurophysiological consequences of ERβ agonism we describe, we investigated the effect of ERβ activation on LTP in the Schaffer collateral pathway of hippocampal area CA1.

Acute hippocampal slices prepared from WT female mice incubated with WAY-200070 (100 nM, 2 h) showed significantly enhanced theta burst stimulation–induced potentiation compared to vehicle-treated slices from the same animals (**Fig. 6a**). Critically, this effect was absent in slices from *Esr2*$^{-/-}$ mice, showing that the effects of WAY-200070 on LTP are ERβ dependent (**Fig. 6b**).

ERβ activation increases dendritic branching and spine number

Estrogen has been shown to alter hippocampal dendritic and spine architecture *in vivo* in association with an enhancement of hippocampus-dependent memory[17]. To examine whether activation of ERβ changes synaptic architecture, we analyzed dendritic branching, spine morphology and spine density in the CA1 and dentate gyrus, regions of the hippocampus where we had previously observed changes in the levels of AMPARs. We dosed OVX rats with WAY-200070 (10 mg kg^{-1}, s.c.) and isolated and fixed brain tissue 48 h later. Sections were then subjected to Golgi staining to visualize the neuronal architecture. We used Sholl analysis (see Methods) to measure the extent of dendritic branching at incrementally greater distances from the cell soma. We observed a significant increase in the number of dendritic intersections in CA1 pyramidal

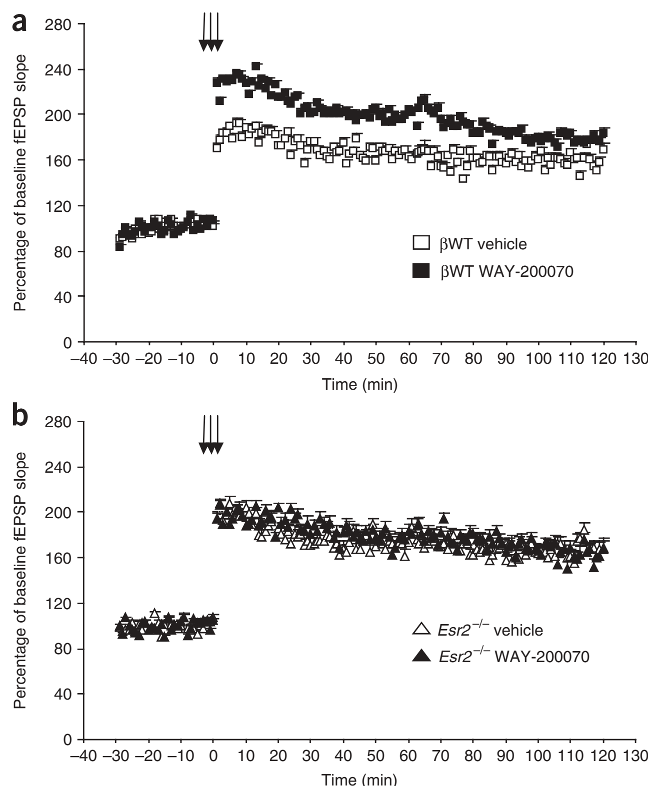

Figure 6 ERβ activation potentiates LTP induced in area CA1 by theta burst stimulation (TBS) of the Schaffer collateral pathway in hippocampal slices from WT mice but not *Esr2*$^{-/-}$. Points represent baseline-normalized fEPSP slopes ± s.e.m. (**a**) WAY-200070 (100 nM) significantly enhanced LTP induced in WT slices compared to vehicle ($P < 0.0001$; derived from covariance parameter, linear mixed model analysis; $n = 9$). (**b**) Incubation of *Esr2*$^{-/-}$ mouse hippocampal slices with the ERβ-selective agonist WAY-200070 (100 nM) had no effect on the magnitude of potentiation. Arrows, TBS.

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neurons at distances of 20–50 μm from the cell body (**Fig. 7a,b**). In the dentate gyrus, there was a significant enhancement in dendritic branching at distances between 170 μm and 230 μm from the soma of granule cells (**Fig. 7c,d**). These data show that ERβ activation increases dendritic branching in distinct regions of the hippocampus *in vivo*.

Excitatory synaptic transmission in the central nervous system takes place mainly at dendritic spines. Spines are classified according to their size and appearance. 'Mushroom' spines (large spine with big head and thick neck) are thought to be very stable, contain the greatest number of AMPARs and have been suggested to act as 'memory spines', whereas thin spines with small heads, which we define as 'lollipop' spines, have been described as being more transient in nature, contain less AMPARs and are hypothesized to act as 'silent synapses', and they consequently are proposed to be the key site for synaptic plasticity[32]. For the purpose of our study, we classified dendritic spines as described in Methods into four types: mushroom (M), lollipop (Ls) and two more categories, nubby (N) and dimple (D). WAY-200070 significantly enhanced the M type of spine on granule cells in dentate gyrus without a significant effect on the other spine types (**Fig. 7e,f**). Furthermore, we did not find significant changes in spine density occurring in other hippocampal regions.

ERβ activation improves spatial memory

We had demonstrated that ERβ can mediate the effects of estradiol on enhanced memory retention in a hippocampus-dependent spatial task (**Fig. 1**). Furthermore we had shown that ERβ activation causes a number of effects at the molecular, physiological and structural level that collectively predict cognitive enhancing activity. To test this hypothesis, we evaluated the effects of WAY-200070 in a hippocampus-dependent spatial memory radial arm maze task.

OVX rats dosed with WAY-200070 (10 mg kg^{-1}, s.c.) showed a significant increase in the percentage of errorless trials on the 'choice trial' as compared to vehicle-treated rats (see Methods). This effect was present at both the 30- and 60-s delay time points between the 'sample' and 'choice' trials. The effect of WAY-200070 was comparable to that of estradiol treatment (**Fig. 8**). In contrast, rats dosed with the ERα agonist (PPT) performed similarly to the vehicle control group, with a marked decrease in the percentage of errorless trials with increasing time delay. Those data suggest that WAY-200070 can mimic the effects of estradiol on a hippocampus-dependent spatial memory task. This effect appears to be mediated through ERβ, as the ERα agonist had no effect in this behavioral paradigm. We obtained similar results for rats using a second cognition paradigm, the Atlantis water maze. The ERβ specific agonist WAY-200070, but not the ERα agonist PPT, improved performance in

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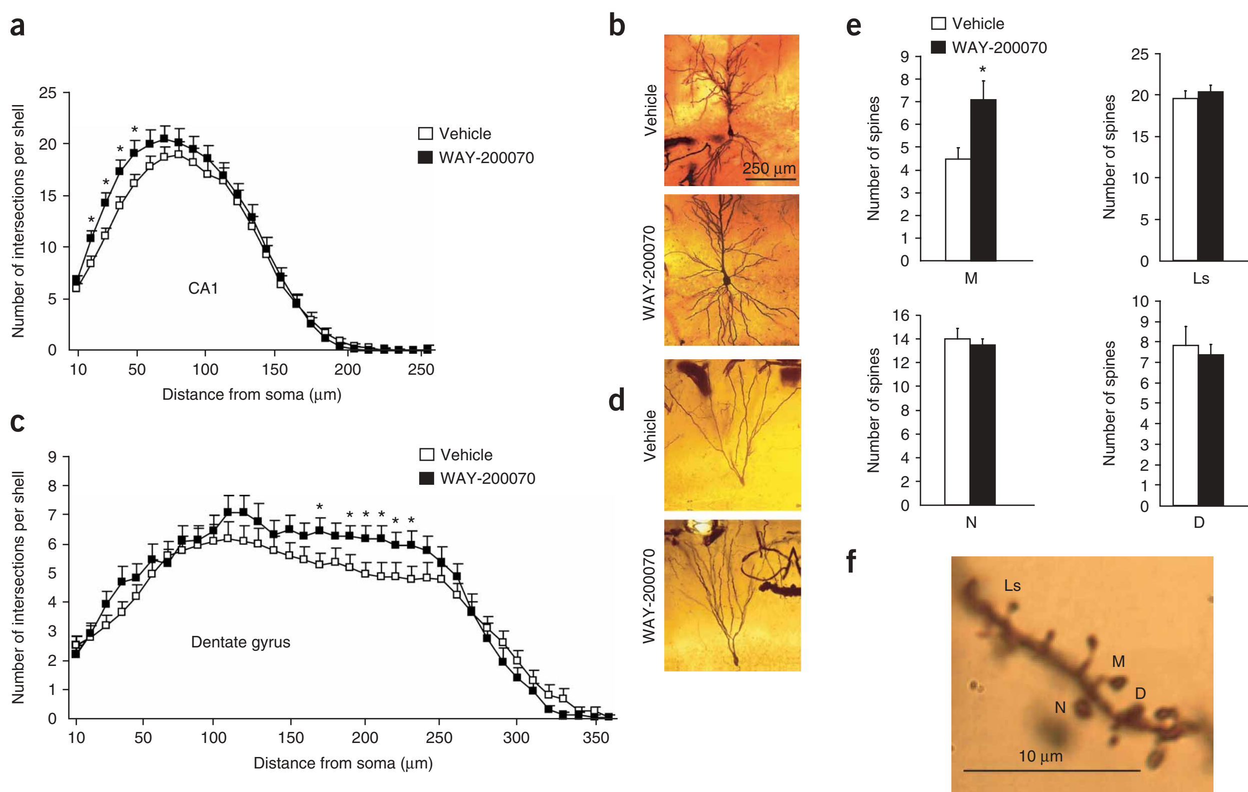

Figure 7 ERβ activation increases dendritic branching and density of mushroom spines in the hippocampus. OVX rats were injected with WAY-200070 (10 mg kg^{-1}, s.c.), and brain samples were collected 48 h later. (**a–d**) Sholl analysis of CA1 pyramidal basilar tree (**a**) and granule cells in dentate gyrus (**c**). $*P < 0.05$ compared to the vehicle-treated rats, $n = 28$ (four animals per treatment, seven neurons per animal). Data presented as mean number of intersections per shell ± s.e.m. (**b**,**d**) Photomicrographs of Golgi staining of the basilar tree of a CA1 pyramidal cell (**b**) and the granule cells of dentate gyrus (**d**) from representative neurons of vehicle and WAY-200070–treated animals. (**e**) Classification of spines as either mushroom (M), lollipop (Ls), nubby (N) or dimple (D) type along 30-μm segments from both the terminal tip region in the most distal portion of the molecular gyrus and the middle of the molecular gyrus. $*P < 0.05$ compared to vehicle-treated samples, $n = 60$ (four animals per treatment, five neurons per animal, three segments per neurons). Data presented as mean number of spines ± s.e.m. (**f**) Photomicrograph showing representative examples of the four types of dendritic spines on granule cells of dentate gyrus.

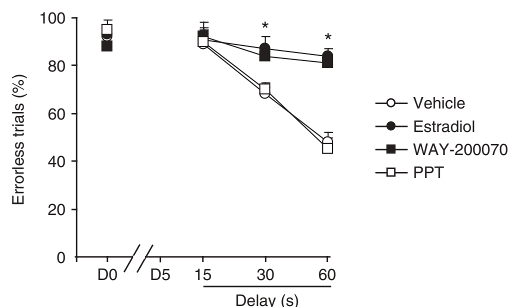

Figure 8 The ERβ agonist WAY-200070, but not the ERα agonist PPT, improves performance on a hippocampus-dependent radial arm maze task. After the last acquisition trial on day 0 (D0), OVX rats were injected with either estradiol, WAY-200070, PPT or vehicle and reinjected 24 h later. Rats were tested on D5 at three time delays (15, 30, and 60 s) in a delayed nonmatch-to-sample task. After significant main effects from ANOVA, *post hoc* comparisons at the 30 and 60 s delays showed increased percentage of errorless trials with estradiol or WAY-200070 treatment (* $P < 0.05$; $n = 20$), whereas PPT treatment was similar to vehicle treatment.

long-term memory formation and memory consolidation (**Supplementary Fig. 3** online).

DISCUSSION

In this study we have combined pharmacological, genetic and behavioral approaches to demonstrate the critical role of ERβ receptors in mediating the effects of estrogen on synaptic plasticity and hippocampus-dependent spatial memory. *Esr2*$^{-/-}$ mice did not show cognitive improvements in a hippocampus-dependent spatial task when dosed with estradiol, in contrast to either *Esr1*$^{-/-}$ and wild-type mice, which both showed enhanced performance (**Fig. 1**). Furthermore, a selective ERβ agonist (WAY-200070) was able to mimic the effects of estradiol in a similar hippocampus-dependent task, whereas an ERα selective compound (PPT) was not (**Fig. 8**). ERβ activation also potentiated LTP in hippocampal slices. This effect was not seen in slices from *Esr2*$^{-/-}$ mice. We have taken further advantage of available selective pharmacological tools and knockout mice to elucidate the underlying molecular mechanism of the observed behavioral and electrophysiological effects. Here we show for the first time that a range of synaptic proteins are elevated in response to *in vivo* dosing with the specific ERβ agonist WAY-200070. Increases in PSD-95, synaptophysin and GluR1 were all observed. The changes in these proteins are consistent with the effects we observed on neuronal architecture and spine number after dosing with WAY-200070. Together our data demonstrate a critical role for ERβ in mediating the effects of estrogen on hippocampally based learning and memory.

We were initially interested in investigating the rapid, 'nongenomic' effects that are engaged following activation of the estrogen receptor[33]. We focused on monitoring levels of phospho-CREB, as activation of CREB has been implicated in learning and memory[34]. Estrogen has been shown to increase CREB phosphorylation within a short timeframe consistent with rapid signaling events[21], both in the brains of estrogen-dosed animals and in cultured primary neurons[21,35]. Similar effects have been reported using a selective ERα agonist applied to either cultured neurons or slices[36]. Our experiments now show that selective ERβ agonists and estradiol induce a significant increase in pCREB in the hippocampus *in vivo* (**Fig. 2b**). In contrast to the published literature, however, animals dosed with the ERα agonist PPT did not show an increase in CREB phosphorylation (**Fig. 2b**). This is despite the fact that PPT was able to bind to and cause translocation of estrogen receptors (**Fig. 2a**). It is possible that the biochemical signaling cascades initiated by ERα activation are very different in the *in vitro* and *in vivo* experimental settings. The ability of an ERβ or ERα agonist to induce CREB phosphorylation may account for the difference in the behavioral end points we report here, in which only an ERβ agonist improved performance (**Fig. 8** and **Supplementary Fig. 3**).

Recruitment of AMPARs containing GluR1 to activated synapses is well established as a mechanism for the long-term maintenance of increased synaptic strength[26]. We found that ERβ activation could increase GluR1 levels in discrete regions of the hippocampus (**Fig. 4a**). Furthermore, our surface biotinylation studies revealed that ERβ activation increased surface levels of GluR1 phosphorylated at Ser845 (**Fig. 5**). These findings are consistent with studies demonstrating that Ser845 phosphorylation is critical for regulating the subcellular trafficking of GluR1 (ref. 31).

We also investigated effects of ERβ activation on PSD-95 because of its role in modulating AMPAR function[27,28]. For example, overexpression of PSD-95 has been shown to increase AMPAR currents by selectively delivering GluR1-containing receptors to synapses[28]. We found that ERβ activation could significantly increase PSD-95 expression levels in the hippocampus (**Fig. 3c**). We speculate that the increased surface expression of GluR1 after ERβ activation (**Fig. 5**) is attributable to a parallel increase in phosphorylation of GluR1 at Ser845 and in PSD-95 expression. Both of these events are known to promote GluR1 trafficking to the surface and increase synaptic strength[27,28,31]. Furthermore, the increase in GluR1 and PSD-95 seems to be specific to ERβ activation because these events are not seen in the *Esr2*$^{-/-}$ mice (**Figs. 3a,c** and **4f**) or after treatment with the ERα specific agonist PPT (**Fig. 3a,c**). To our knowledge, this is the first time that increases in GluR1 and PSD-95 have been reported in response to ERβ agonism *in vivo*.

Recruiting more GluR1-containing AMPA receptors to activated synapses is known to be critical for LTP[26]. It has been reported that GluR1 null mice are deficient in hippocampal CA3-CA1 LTP[37] and show a profound impairment in spatial memory[38]. In previous studies, we had shown that *Esr2*$^{-/-}$ mice have deficits in their expression of LTP in the CA1 region as well as compromised hippocampus-dependent memory[23]. Here we have demonstrated that ERβ activation can potentiate CA3-CA1 LTP in wild-type but not *Esr2*$^{-/-}$ mice (**Fig. 6**). Our findings further implicate ERβ as a modulator of hippocampal synaptic plasticity. Estradiol has been previously reported to increase expression of mRNA for NMDAR subunit NR2B, the number of NR2B binding sites, and the synaptic localization of NR2B-containing receptors[18,19,39]. In our studies, neither estradiol, the ERβ selective agonists (WAY-200070 and WAY-202779), nor the ERα selective agonist PPT had an affect on the expression of NMDAR subunits NR2B and NR1 (**Figs. 3d** and **4d**). We attribute our inability to see an increase in NR2B expression to substantial experimental differences. For example, our *in vivo* studies were conducted four hours after a single injection of the ERβ agonist (**Fig. 3**), whereas previous studies were conducted after a 14-d injection regimen of estradiol (refs. 18,40). Although our present study did not detect changes in NR2B or NR1 protein expression, we do not rule out NMDAR contributions to ERβ-mediated synaptic activities. Indeed, it is interesting to speculate that the changes seen here are required to drive subsequent changes in NMDAR expression.

Estrogen has been reported to promote the formation of new dendritic spines and excitatory synapses in the hippocampus[16,17]. Notably, we observed that the ERβ agonist induced a series of morphological changes in CA1 and dentate gyrus, not previously reported with estradiol treatment. First, ERβ activation increased dendritic branching in CA1 pyramidal neurons (**Fig. 7a,b**) and dentate

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gyrus granule cells (**Fig. 7c,d**). This same treatment produced a significant increase in the 'mushroom' type of dendritic spine on the granule cells in dentate gyrus without effects on total spine number. These findings are reminiscent of an earlier study wherein estrogen was shown to have no effect on total spine number but to increase mushroom spines on CA1 pyramidal neurons in OVX mice[17]. The increase in the large mushroom spines is consistent with our own data on increased GluR1 levels after ERβ activation and a recent report of spine size being determined by the synaptic insertion of GluR1 (ref. 41).

We have demonstrated in this report that ERβ activation can enhance spatial memory in both the radial arm maze and Atlantis water maze in a manner similar to that seen with estradiol (**Fig. 8** and **Supplementary Fig. 3**). These findings build on previous reports demonstrating an effect of estradiol in cognition and suggest that ERβ may be particularly important in this regard. The ability of estrogen, or of more selective compounds, to modulate synaptic physiology and cognition at different ages is currently an area of much debate, in particular in the area of hormone replacement therapy (HRT). Recent results from the Woman's Health Initiative Memory Study (WHIMS) suggest that age of HRT treatment onset is critical for producing a positive effect on cognition[42]. If HRT is received early (aged 56 years or earlier, or within 5 years of hysterectomy) cognitive performance is improved compared to either older HRT-treated women or untreated women. Furthermore recent studies have reported that estrogen may also have beneficial effects in a variety of other diseases, including Alzheimer's disease[43] and schizophrenia[44].

Our findings elucidate a new ERβ-mediated mechanism by which estrogen may influence synaptic plasticity in the hippocampus and ultimately learning and memory. Activation of this pathway may confer some of the CNS-mediated benefits of estrogen without the feminizing side effects and may offer a new therapeutic approach for diseases with cognitive deficits such as Alzheimer's disease and schizophrenia.

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METHODS

Compounds. WAY-200070 and WAY-202779 were synthesized by the medicinal chemistry group at Wyeth. PPT was purchased from Tocris. Estradiol was purchased from Sigma.

Animals. Long Evans OVX female and male rats were obtained from Charles River Laboratories. *Esr1*$^{-/-}$ or *Esr2*$^{-/-}$ and their WT (αWT and βWT) littermates with a C57/BL6N background were generated at Wyeth[23]. Animals were maintained on a 12-h light-dark cycle with food and water *ad libitum*. Maintenance and research were conducted in accordance with the US National Institutes of Health Committee on Laboratory Animal Resources policies and guidelines for handling and used of laboratory animals and approved by the institutional animal care and use committee at Wyeth Research.

Immunoblotting. The following primary antibodies were used: Rabbit anti-GluR1, rabbit anti–Ser845-phospho-GluR1 and rabbit anti-synaptophysin (Chemicon International, 1:1,000); rabbit anti-CREB, rabbit anti–Ser133-phospho-CREB and rabbit anti–PSD-95 (Cell Signaling, 1:1000); goat anti-NR2B (Santa Cruz Biotechnology, 1:1000); and rabbit anti-actin (Sigma, 1:10,000). Equivalent amounts of protein were resolved by 4–12% SDS-PAGE gel and transferred to nitrocellulose membrane. The membranes were blocked for 1 h in TBS containing 0.1% Tween-20 and then incubated with the primary antibody of interest overnight at 4 °C followed by incubation with horseradish peroxidase–linked secondary antibodies—goat antibody to rabbit immunoglobulin or donkey antibody to goat immunoglobulin (Jackson ImmunoResearch, 1:10,000) and developed using enhanced chemiluminescence. Densitometric analysis of western blots was conducted using a Bio-Rad GS-710 Calibrated Imaging Densitometer and quantified using Quantity One version 4.1.0.

Immunohistochemistry. Free-floating double-labeling peroxidase-based immunohistochemistry was performed in the same manner as reported previously[45]. Sections were incubated with one of the following primary antibodies: rabbit anti-GluR1, 1:5,000; rabbit anti-NR1, 1:100; mouse anti-synaptophysin, 1:20,000; or mouse anti–PSD-95, 1:5,000 (all from Chemicon International). Sections were visualized with an Olympus BX51 microscope and the optical density was analyzed using MetaMorph software (Universal Imaging Corporation).

Biochemical measurement of surface-expressed receptors. Briefly, 300-μm hippocampal slices were prepared from OVX Long Evans female rats. Slices were equilibrated in oxygenated ACSF buffer (125 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.5 mM $CaCl_2$, 1.5 mM $MgSO_4$ and 1.25 mM KH_2PO_4) for 15 min. followed by a 60-min recovery period at 30 °C. Slices were treated with 10 nM WAY-200070 in 0.05% DMSO in oxygenated ACSF buffer for a further 60 min. After treatment, surface biotinylation reactions were performed as described[46] with the following modifications. Slices were incubated with ACSF containing 1 mg ml^{-1} sulfo-NHS-LC-biotin (Pierce) for 45 min on ice. We then removed 25 μg of protein to measure total GluR1. Bound proteins were resuspended in 50 μl of SDS sample buffer and boiled. Immunoblotting was performed on both total and biotinylated (surface) proteins. Total GluR1 expression was normalized to actin and surface GluR1 immunoreactivity was normalized to total.

[^{3}H] estrogen receptor assay. Binding assays were performed as described[47]. Briefly, brain tissues were homogenized and nuclei were isolated by differential centrifugation. Nuclei were incubated with [^{3}H]estradiol overnight. In all experiments, specific binding was calculated by subtracting nonspecific binding from total binding, the results were expressed as femtomoles bound per milligram protein.

Quantification of dendritic branching, spine density and spine type. Fixed coronal blocks of rat tissue from the hippocampus were stained using the Rapid Golgi protocol as previously described[48]. We quantified dendritic branching in hippocampal CA1 pyramidal cells and dentate gyrus granule cells was quantified. Specifically, we randomly selected seven cells from four brains in each group for dendritic branching analysis of their basilar dendritic tree. Camera lucida drawings were made of the soma and basilar dendritic arbor and were quantified using Sholl analysis. The numbers of dendritic branches intersecting with concentric circles of equidistant increasing diameters were counted out to 250 or 350 μm from the soma (each circle was equivalent to a 10-μm increment, with the soma at the center) and compared statistically by unpaired *t*-test ($P < 0.05$).

Dendritic spine morphology and density were evaluated from dentate gyrus granule cells and CA1 pyramidal cells. Using Zeiss brightfield research microscopes, spines were counted at ×1,600 magnification from two regions of the granule cells: along the terminal tip segments in the distal (outer) region of the molecular layer and on segments located in the middle third of the molecular layer. We used four categories of spines: 'M-type' (mushroom), characterized by a large, well defined spine head and a thick spine neck; 'Ls-type' (lollipop or small head), characterized by a well defined spine head and a very thin spine neck; 'N-type' (nubby), characterized by a poorly defined spine head and a thickened spine neck; and 'D-type' (dimple), characterized by a poorly defined spine head and the absence of a definitive spine neck. Similar spine configuration categories have been used previously[49]. For each neuron, we counted spines (and assigned their configurations) along 30-μm segments, using three segments from each neuron region in the study (for example, three segments from the terminal tips of the granule cell and three segments from the middle portion of the granule cell). The numbers of spines for each configuration were statistically compared between drug-treated and vehicle-treated animals using the unpaired *t*-test ($P < 0.05$).

Hippocampal slice preparation and electrophysiology. Detailed methods were as previously described[23]. In brief, for each recording session, dorsal hippocampal slices were prepared from both *Esr2*$^{-/-}$ mice and their wild type (βWT) counterparts. Slices from each individual animal were then split between two recording chambers, one of which was perfused with ACSF containing WAY-200070 (100 nM), the other containing ACSF with vehicle (0.01% DMSO). Thus, each experiment generated parallel field recordings from hippocampal slices representing each of four treatment groups. Treatment group recordings

were randomized across different chambers between experiments. Once in their respective chambers, slices were allowed to equilibrate for at least 60 min before beginning the recording session. Micropipettes filled with ACSF (2–5 MΩ impedance) were lowered into the dendritic layer of area CA1, while platinum concentric bipolar stimulating electrodes were lowered onto the immediately adjacent dendritic surface (Schaffer collaterals). For each slice, an input-output curve was generated using constant current (1 to 100 μA) stimulating pulses of 200 μsec duration, after which the stimulus current was adjusted such that the slope of the evoked field excitatory postsynaptic potential (fEPSP) stabilized at about 40% of maximum. Following establishment of the submaximal baseline, LTP was induced with a conditioning stimulus consisting of three theta burst trains delivered 20 s apart. Each theta burst train itself consisted of ten 5-Hz series of four 100-Hz pulses of 400 μsec duration.

Y-maze. A Y-maze constructed of Plexiglas and consisting of three alleys radiating at a 45° angle was placed in a pool of water (23 ± 2 °C) and used in these studies. One alley was designated the start alley and the other two were suitable for escape through platforms at the end of the alley. After 2 d of habituation to the Y-maze, animals (wild type or knockout, 20–25 g body weight, $n = 20$) were trained on four consecutive days, with ten trials per training session. A trial consisted of a sample run in which one alley was closed off, forcing the animal to swim to the open alley. After allowing for a 20-s rest on the platform, we began the choice run, in which the animal could swim to either alley. Escape was possible through the alley that was previously blocked. Animals were allowed to find the platform or were gently steered in that direction after 60 s. The order of arms available in the sample run was counterbalanced across the 10 trials. After the last acquisition trial, mice were injected with 5 μg of estradiol (0.1 ml, s.c.) or a 5% ethanol, 95% miglyol oil vehicle, with a repeat injection 24 h later. Five days after the first injection, each animal received five trials at each of three time delays: 30, 60, and 120 s. After a sample run, the mouse was removed and placed in a holding cage for the duration of the delay interval, after which the choice run was executed. The order of the arms available in the sample run was counterbalanced, and the order of time delays was randomly assigned across the 15 trials.

Radial arm maze. OVX female Long–Evans rats ($n = 40$, 200–250 g body weight) were placed on a water-deprivation schedule, with access to water for 30 min per day. Rats were placed in the center of the maze with access to all eight arms. Each arm was baited with 100 μl of water in a recessed cup at the end of the runway. Habituation was conducted for 15 min on each of five consecutive days. Animals were trained in five trials per day on three consecutive days. Each trial began by placing the animal in the center of the maze with all doors closed for a 10-s period, after which access to one of the alleys was permitted. After consumption of the water and exit from the arm, the gate was closed, briefly trapping the animal in the center of the maze; 2 s later, the original gate and a new gate were opened simultaneously. A correct response was noted when the animal entered the novel arm, thus receiving another reinforcement. After the last acquisition trial, rats received two s.c. injections, separated by 24 h, of 0.2 ml of one of the following treatments: estradiol (20 μg), PPT (10 mg kg^{-1}), WAY-200070 (10 mg kg^{-1}), or 5% ethanol, 95% miglyol oil vehicle.

For the delayed nonmatching-to-sample test, all tests were conducted 5 d after the first injection. The test consisted of two consecutive sample trials and one choice trial. In the first sample trial, the animal was given access to a single arm of the maze, allowed to consume the water and to return to the central compartment. Immediately thereafter, the second sample trial was initiated by giving the animal access to another arm of the maze. Again, the animal was allowed to consume the water, and upon its return to the central compartment, all guillotine doors were closed, capturing the animal within it. After a delay of 15, 30, or 60 s, a choice trial was executed, in which the animal had access to three arms, two from each of the sample trials and a novel, as-yet-unvisited arm. All animals were tested five times at each of the three delay intervals. The arms used in the sample and choice trials were determined randomly, as were the intervals used in each test.

Atlantis water maze. A 1.5-m diameter white fiberglass pool with a depth of 0.6 m was used. The pool was filled with water to a depth of 0.3 m that was made opaque with the addition of 150 ml tempera paint. The pool was situated in a room that contained a variety of two- and three-dimensional distal cues. The swim paths of the rats were tracked using a video camera suspended centrally above the pool and all sessions were recorded on videotape. Data were collected and analyzed online using Actimetrics Water maze software. Male Long-Evans rats ($n = 12$) were pretrained for 1 d (four trials per day) in the water maze with a visual cue task in which the distal extra-maze cues were occluded by curtains around the circumference of the pool. The escape platform was concealed from view 1 cm below the surface of the water and the platform position was identified by a visual cue suspended above the escape location. After visual cue training, all rats received 4 d of spatial training (four trials per day) with the curtains removed and the distal extra-maze cues visible. Each rat was placed in the pool facing the wall at one of the start locations (N, S, E, W). Using the Atlantis platform (15 cm diameter), rats were trained to dwell within a 35 cm diameter zone above the platform location for a predetermined period of time. This activated the platform, which was then automatically released from the bottom of the pool, allowing the rat to escape onto it. The Atlantis platform was activated after 1 s dwell time on day 1, 2 s on day 2, and 3 s on days 3 and 4. Rats were given a maximum of 120 s to find the platform in all trials. If the rat had failed to activate the platform within 90 s, the platform would rise. Once on the platform, rats remained there for 30 s before being removed by the experimenter. After spatial training a single probe test was conducted 5 d later.

Statistical analysis. For all biochemical experiments, data were analyzed either by Student's unpaired *t*-test or two-way ANOVA followed by *post hoc* analysis. For the Y-maze, data were analyzed in a mixed-design ANOVA, with strain and drug treatment as between-group variables and the delay interval as the within-group variable. Separate ANOVAs were conducted to investigate the effects of estradiol on acquisition rates in *Esr1*$^{-/-}$ and *Esr2*$^{-/-}$ strains. Further tests were conducted using planned *post hoc* simple effects contrasts ($P < 0.05$). For analysis of the radial arm maze, data were expressed as the percentage of errorless trials per training session and were analyzed in a mixed design two-way ANOVA with drug treatment as the between group variable, and the delay interval as the within group variable. *Post hoc* analysis consisted of simple contrast test ($P < 0.05$).

Note: Supplementary information is available on the Nature Neuroscience website.

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