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Research report

17β-estradiol (E2) promotes growth and stability of new dendritic spines via estrogen receptor β pathway in intact mouse cortex

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ABSTRACT

The steroid hormone 17β-estradiol (E2) remodels neural circuits at the synaptic level in the mammalian hippocampus and cortex. However, the underlying mechanism of synapse dynamics remains unclear. To elucidate the mechanism, we traced individual dendritic spines on layer V pyramidal neurons of the primary sensory cortex in adult female mice under E2 intervention using two-photon *in vivo* imaging microscopy. We confirmed the increase of the spine density upon E2 treatment in the intact mouse cortex. Furthermore, we found that this increase is due to the promotion of spine formation and the stability of newly formed spines. E2 treatment doesn't alter the elimination rate of pre-existing spines. Our results also indicate that the activation of the estrogen receptor β (ERβ) mimics the effects of E2 administration on spine dynamics. Taken together, our findings suggest that estrogen promotes growth and stability of new dendritic spines via the ERβ pathway in the intact cortex of female mice.

1. Introduction

Steroid hormones not only exert regulatory effects in multiple organs and tissues, but also have considerable importance in the mammalian brain function (Brann et al., 2007; Marinho et al., 2008), including processing and storage of new information (Hayashi and Majewska, 2005; Matus, 2005; Holtmaat and Svoboda, 2009; Harris et al., 2002) as well as learning and memory (Phan et al., 2012). The actions of estrogens at diverse neural systems, such as the cerebral cortex, basal forebrain, hippocampus and striatum, mediate cognitive functions (Luine and Frankfurt, 2012). For example, the prefrontal cortex is considered as an important region of action for the cognitive effects of estrogen (Keenan et al., 2001). With the discovery of hippocampal steroids mediating neuronal structure and behavior over three decades ago (Adams et al., 2001; Ogawa et al., 2003; Gresack and Frick, 2006), the impact of estrogens, such as 17β-estradiol (E2), revealed to be far reaching on the central nervous system (Cooke and Woolley, 2005). Numerous, replicated findings have demonstrated that the manipulation of E2 levels can alter dendritic arbors and neuronal function in diverse cortical regions and hippocampus in *in vitro* brain slices (Moult and Harvey, 2008; Yankova et al., 2001; Woolley and McEwen, 1994; Chen et al., 2009; Tuscher et al., 2016; Sellers et al., 2015; Galvin and Ninan, 2014; Khan et al., 2013). E2 exposure increases the spine density in the medial prefrontal cortex (Tuscher et al., 2016; Khan et al., 2013; Hao et al., 2006), the primary cortex (Chen et al., 2009; Sellers et al., 2015; Khan et al., 2013), and hippocampus neurons (Phan et al., 2012; Tuscher et al., 2016; MacLusky et al., 2005; Gould et al., 1990). Moreover, it has been reported that acute E2 treatment rapidly induces spinogenesis via the estrogen receptor β (ERβ) pathway in cultured cortical neurons of rats (Sellers et al., 2015). E2 mediated-enhancing effects on spinogenesis in the cerebral cortex may be associated with E2 enhancement of cortical functions, such as executive function and sensory processing (Khan et al., 2013). But the underlying neuroplasticity mechanisms of E2 enhancement of spinogenesis remain unclear.

Dendritic spines are tiny postsynaptic protrusions which receive the majority of the input signals of excitatory synapses. Spine dynamics are an ideal indication of synapse plasticity (Yang et al., 2009; Xu et al., 2009). Long-term coordinated spine modifications result in the refinement of neuronal circuits and changes in the function of synaptic

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connections. Therefore, these spine modifications are considered essential for advanced cognitive function (Chen et al., 2012; Jung et al., 2008). Although a large number of studies have reported that E2 increases the total spine number in diverse brain regions, the underlying mechanism of the spine dynamics remains unclear. To be precise, the final change of the total spine number can be caused by the different fates of spines, including pre-existing spines, newly formed spines, and eliminated spines. For instance, both the increase in spine formation and decrease in spine elimination could contribute to the net growth of the total spine number. Previous *in vitro* researches have demonstrated that E2 increases the total spine number through group comparison, but hasn't got the underlying spine dynamics contribute to the changes. Although it has been reported that acute E2 treatment triggered new spine generation without affecting the original spine pruning (Mendez et al., 2011), the long-term impact of E2 on spines is still unclear. To reveal the impact of E2 on the regulation of synapse plasticity in detail, we need to trace the fate of individual spines on the same living animal.

The combination of the transgenic technique (Feng et al., 2000) and two-photon laser scanning microscopy imaging (Helmchen and Denk, 2005; Svoboda and Yasuda, 2006) enables researchers to track the same apical dendrite spine in the intact cerebral cortex (Grutzendler et al., 2002; Trachtenberg et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005; Cruz-Martin et al., 2010). *Thy1*-GFP-M transgenic mice with sparse labeling of their pyramidal neurons with green fluorescent protein (GFP), predominantly in layer V of the cortex, are used widely in *in vivo* studies of synaptic plasticity (Feng et al., 2000). In the present study, we assessed the role of E2 modifying the cortical synaptic plasticity by using transcranial, two-photon microscopy to track the dynamics of individual spines on layer V pyramidal neurons in the sensory cortex of ovariectomized female *Thy1*-GFP-M transgenic mice. We also revealed the receptor–pathway mechanisms using estrogen receptor agonists. The findings indicate that in the intact sensory cortex, the activation of the ERβ pathway increases the spine density through promoting growth and stability of new dendritic spines.

2. Results

2.1. Timeline of experiment and spine identification

In this study, we set the first imaging day to day 0. Adult *Thy1*-GFP-M female mice were subjected to a ten-day recovery phase after an open-skull operation (surgical operation usually at 60 d postnatal, ~ P60) on the sensory cortex region (Fig. 1A) and received an ovariectomy (OVX) subsequently. Mice were then divided randomly into two groups and immediately imaged *in vivo* by two-photon laser scanning microscopy at ~P85d (Fig. 1B). All animals were imaged three times in a four-day interval (Fig. 1B). We determined the effects of 17β-estradiol (E2), a commonly used gonadal steroid, on the spine dynamics of the intact cortex. E2-release pellets (1.5 mg/pellet, 21 d) were once implanted on the lateral side of the neck between ear and shoulder immediately after the first *in vivo* imaging (day 0).

In the present study, we identified the seven kinds of spines based on the presence or absence of spines at the three times points (day 0, 4 and 8; Fig. 1C). We especially focused on newly formed, pre-existing, and lost spines to analyze their fates after E2 treatment and specifically traced and analyzed the changes in the ratio of persistent spines, lost-return spines, and persistent newly formed spines.

2.2. Effects of E2 treatment on turnover of dendritic spines in the sensory cortex

To investigate whether and how E2 modulates the turnover of dendritic spines in the intact cortex, we used two-photon laser scanning microscopy to record changes in the spine dynamics upon E2 treatment. We repeatedly imaged and tracked the same group of dendritic spines before and after the initial pharmacological intervention. We found no significant change in the spine elimination rate upon E2 treatment. However, the spine formation rate increased significantly four days after E2 treatment (Fig. 2B; spine formation during days 0–4: control, 4.6 ± 0.6%; E2, 6.4 ± 0.5%, ($P = 0.034$, Student's t-test)). The increase in the spine formation rate was still obvious eight daysafterE2 exposure. It is notable that the above increase in the spine formation rate primarily occurred during the first four-day interval rather than the second one (days 4–8). No change in the spine elimination rate, but increase in the spine formation rate resulted in a significant increase in the total spine number in E2 implanted mice compared to that in control mice (Fig. 2C; total spine number during days 0–4: control, 96.5 ± 0.9%; E2, 100.0 ± 0.5%, ($P = 0.006$, Student's t-test); total spine number during days 0–8: control, 93.6 ± 1.6%; E2, 98.9 ± 1.2%, ($P = 0.024$, Student's t-test)).

2.3. Influence of E2 treatment on the fates of different spines

The net increase of the total spine number may be due to several reasons, including the decrease in the pre-existing spine elimination rate, the increase in the lost-spine return rate, and/or the increase of the newly formed spine survival rate. In order to uncover the underlying dynamic mechanism, we analyzed and compared the ratios of persistent spines, lost-return spines, and persistent newly formed spines, respectively, between E2 treatment group and control. Results revealed no significant difference in the persistent spine ratio and the lost-return spine ratio, which suggests that E2 treatment has no effect on the pre-established network and circuits pruning. However, in the E2 exposure group, the persistent newly formed spine ratio is almost twice as high as that in the control group (Fig. 3C; control, 37.5 ± 9.0%; E2, 68.8 ± 3.9%, ($P = 0.016$, Student's t-test)). Therefore, in addition to the enhanced formation of spines, the significant increase of persistent newly formed spines also contributes to the increase in the total spine number in the cortex of E2-exposed mice.

2.4. Effects of the E2 pathway on spine dynamics

In the mouse cerebral cortex, mainly two kinds of ERs, ERα and ERβ, are present (Sharma and Thakur, 2006; Mitra et al., 2003; Perez et al., 2003). To investigate which ER pathway is involved in the E2-mediated neural circuit remodeling, we subcutaneously injected specific agonists, α-PPT and β-DPN, to activate ERα or ERβ separately. The results showed that neither α-PPT nor β-DPN modified spine elimination. Nevertheless, we found that β-DPN intervention, but not α-PPT, increased the spine formation rate in the sensory cortex. (Fig. 4B; spine formation during days 0–4: control, 5.2 ± 0.9%; β-DPN, 9.3 ± 1.0%, ($P = 0.010$); spine formation during days 0–8: control, 5.5 ± 0.5%; β-DPN, 10.4 ± 1.0%, ($P = 0.001$); statistical analysis by Student's??-test). No change in spine elimination and increase in spine formation in the β-DPN group mimic a significant increase in the total spine number induced by E2 (Fig. 4C; total spine number during days 0–4: control, 97.6 ± 1.1%; β-DPN, 102.8 ± 0.8%, ($P = 0.002$); total spine number during days 0–8: control, 95.8 ± 0.9%; β-DPN, 102.6 ± 1.2%; ($P = 0.001$); statistical analysis by Student's t-test).

Moreover, we analyzed the effects of β-DPN on the spine dynamics in detail and found that just like the E2 treatment, β-DPN changed neither the persistent spine ratio nor the lost-return spine ratio. However, β-DPN increased the persistent newly formed spine ratio significantly. (Fig. 5C; control, 39.5 ± 3.5%; β-DPN, 52.5 ± 4.2%, ($P = 0.034$; statistical analysis by Student's t-test)). The results indicate that E2 influences the spine dynamics through the ERβ pathway.

3. Discussion

Through *in vivo* imaging study we found that E2 enhances the level of the total spine number in the intact sensory cortex due to the significant increases of not only the formation rate but also the survival

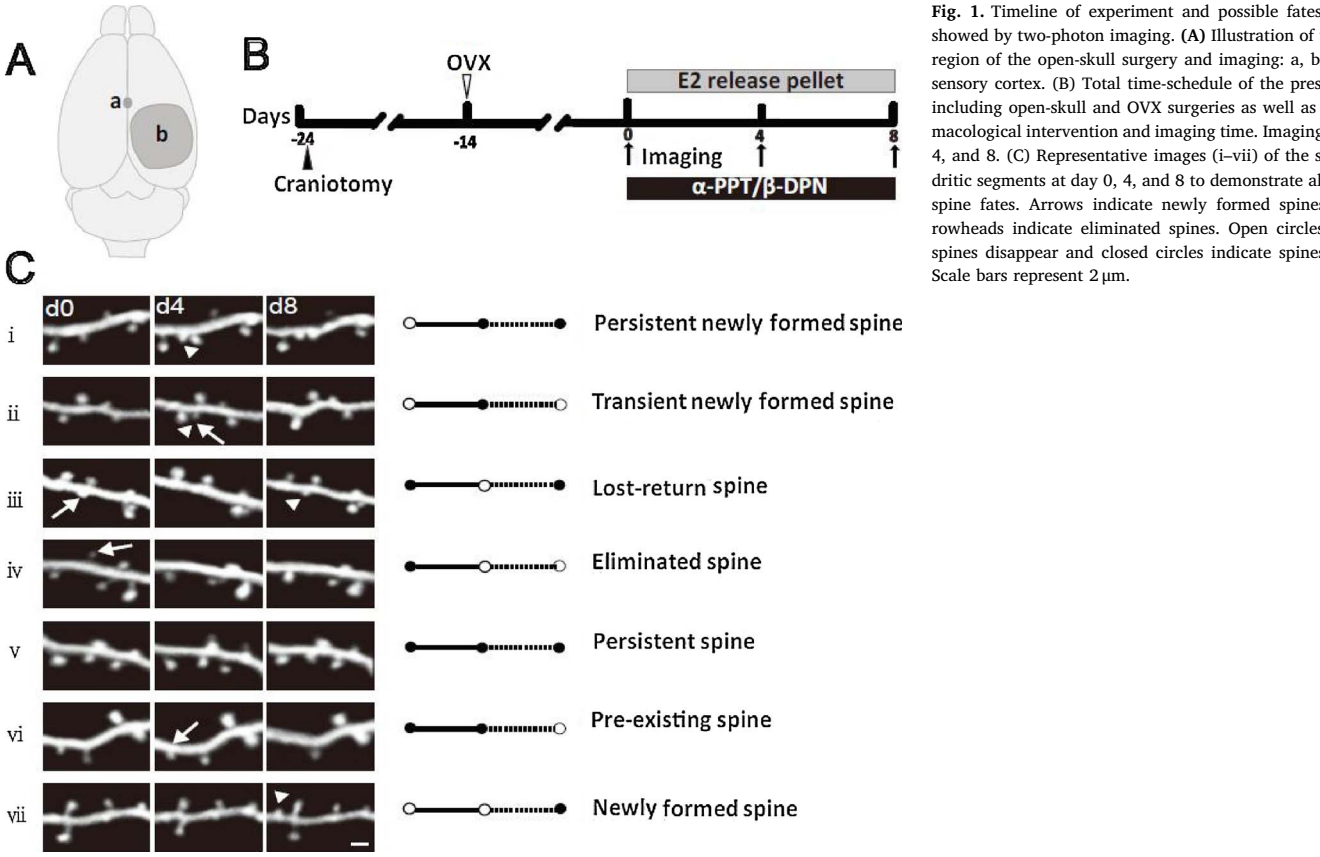

Fig. 1. Timeline of experiment and possible fates of spine showed by two-photon imaging. **(A)** Illustration of the target region of the open-skull surgery and imaging: a, bregma; b, sensory cortex. (B) Total time-schedule of the present study including open-skull and OVX surgeries as well as the pharmacological intervention and imaging time. Imaging at day 0, 4, and 8. (C) Representative images (i–vii) of the same dendritic segments at day 0, 4, and 8 to demonstrate all possible spine fates. Arrows indicate newly formed spines and arrowheads indicate eliminated spines. Open circles indicate spines disappear and closed circles indicate spines present. Scale bars represent 2 μm.

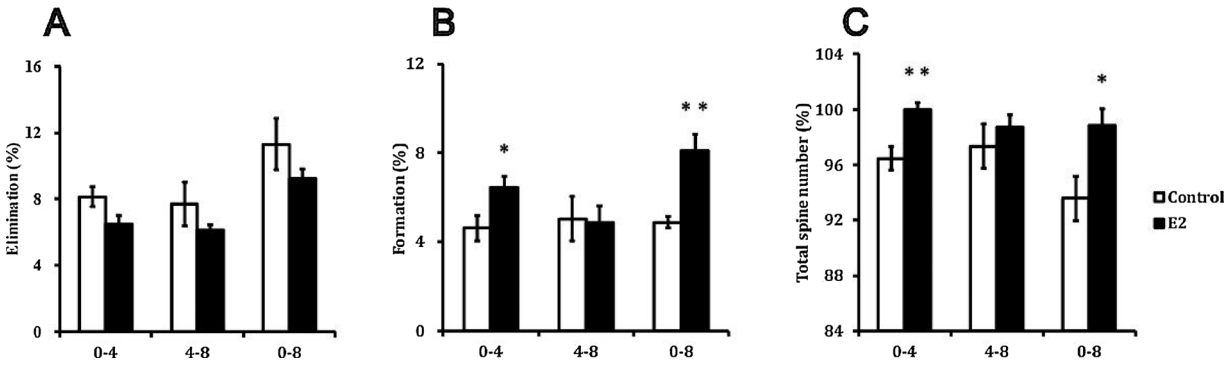

Fig. 2. Regulation of E2 on spine turnover and density in the intact sensory cortex (statistical analysis by Student's??-test). (A) Percentage of spine elimination during days 0–4 in control (n = 7) and E2 (n = 9) mice ($P > 0.05$); Percentage of spine elimination during days 4–8 in control (n = 6) and E2 (n = 9) mice ($P > 0.05$) and during days 0–8 in control (n = 6) and E2 (n = 9) mice ($P > 0.05$). (B) Percentage of spine formation during days 0–4 in control (n = 7) and E2 (n = 9) mice ($^{*}P < 0.05$), during days 4–8 in control (n = 6) and E2 (n = 9) mice ($P > 0.05$), and during days 0–8 in control (n = 6) and E2 (n = 9) mice ($^{**}P < 0.01$). (C) Percentage of spine density during days 0–4 in control (n = 7) and E2 (n = 9) mice ($^{**}P < 0.01$), during days 4–8 in control (n = 6) and E2 (n = 9) mice ($P > 0.05$), and during days 0–8 in control (n = 6) and E2 (n = 9) mice ($^{*}P < 0.05$).

rate of new spines. In addition, E2 treatment didn't alter the fates of lost and pre-existing spines. Furthermore, our results suggest that E2 promotes the growth and stability of new dendritic spines via the ERβ pathway in the sensory cortex. To the best of our knowledge, this is the first report demonstrating effects of E2 and estrogen receptor agonists on the synaptic plasticity in the intact mouse cerebral cortex.

In recent years, memory processing of estrogens in the brain has received increasing interest (Choleris et al., 2008; Choleris et al., 2009). Estrogen treatment improves the spatial working memory in the T-maze (Fader et al., 1998; Miller et al., 1999), radial arm maze (Bimonte and Denenberg, 1999; Luine et al., 1998), and water maze (O'Neal et al., 1996; Sandstrom and Williams, 2001; Sandstrom and Williams, 2004); the non-spatial memory in object recognition and avoidance tasks (Farr et al., 1995; Gresack and Frick, 2004; Vaucher et al., 2002); as well as the spatial reference memory in the Morris water maze (Heikkinen et al., 2002; Rissanen et al., 1999). It is well established that E2 is involved in the modulation of not only the memory but also the synaptic plasticity. The interest in the synaptic plasticity mechanisms of E2 for enhancing the memory has been increasing since neural circuit plasticity has been discovered as a powerful mechanism for information storage. Very recent evidence has shown that circulation changes of estradiol alter the dendritic spine density on pyramidal cells in either the cortex or the hippocampus, both of which are closely related to cognition, as a high spine density is correlated with high estrogen levels (Chen et al., 2009; Khan et al., 2013; Gould et al., 1990; Woolley et al., 1990; Luine and Frankfurt, 2013; Tang et al., 2004; Inagaki et al., 2012). E2 administration has repeatedly been shown to increase the density of dendritic spines in cortex and hippocampus (Mukai et al.,

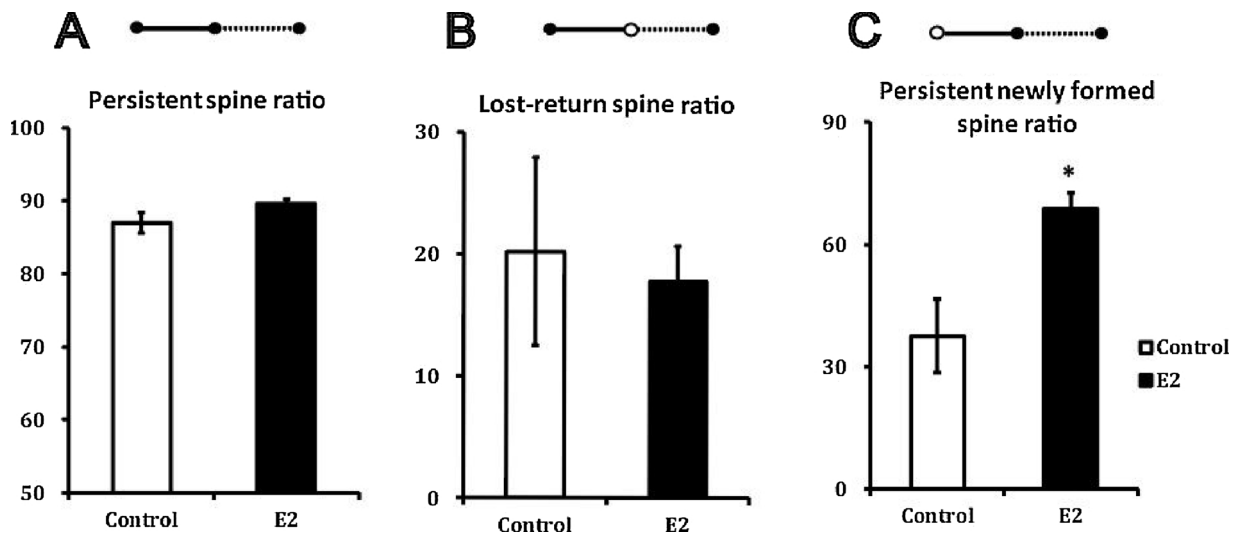

Fig. 3. Regulation of E2 on the fates of different spines in cortex (statistical analysis by Student's t-test). **(A)** Persistent spine ratio corresponding to the fate of pre-existing spines in control (n = 6) and E2 (n = 9) mice ($P > 0.05$). (B) Lost-return spine ratio corresponding to the fate of lost spines in control (n = 6) and E2 (n = 9) mice $P > 0.05$). **(C)** Persistent newly formed spine ratio corresponding to the fate of newly formed spines in control (n = 6) and E2 (n = 9) mice (*$P < 0.05$).

2007; Srivastava et al., 2008). In the brain, the actions of E2 are mediated by estrogen receptors (ER), mainly including estrogen receptor α (ERα) and estrogen receptor β (ERβ). The expression of ERs in the brain has been long acknowledged: ERα and ERβ are both expressed in the hippocampus of rats, mice, and primates (Zhou et al., 2014). ERα is highly expressed in the mammalian forebrain, whereas ERβ is highly expressed in the cortex and the hippocampus (Mitra et al., 2003; Milner et al., 2001; Milner et al., 2005; Kritzer, 2002). Previous studies indicated that the activation of ERα regulates sexual behaviors and reproductive neuroendocrine function (Wersinger et al., 1997; Lindzey et al., 1998), whereas ERβ modulates various brain functions, such as learning and memory (Jacome et al., 2010). Investigations of the underlying modulation of the total spine number through different estrogen agonist/antagonist receptors have identified divergent roles for ERα and ERβ in hippocampus and cortex. In hippocampal neurons, activation of ERα, but not ERβ, induces an increase in spine density (Mukai et al., 2007), whereas activation of ERβ, but not ERα, has been shown to increase the spine density in cortical neurons (Srivastava et al., 2010). Our knowledge about the effects of E2 or ER on spine plasticity has come predominantly from studies in brain slices, fixed brain tissues or live neuronal cultures, and mainly limited in the changes of spine density. For full understanding of synaptic dynamics change induced by E2, it is crucial to trace the fate of individual spines directly, in the living, intact brain. The results from our *in vivo* imaging study not only confirmed that E2 induces the increase in spine density through ERβ pathway but also revealed that this increase is due to the promotion of spine formation rate and selective stabilization of newly formed spine in intact sensory cortex, which demonstrates the complexity of E2 roles on the rewiring of neural circuits.

Recent studies have begun to reveal the molecular mechanisms underlying the increase of dendritic spines induced by estrogens. Estradiol has been shown to regulate actin cytoskeletal dynamics, a key mechanism underlying the dynamics of the spine structure, in the hippocampus by stimulating the RhoA kinase (ROCK) pathway (Kramar et al., 2009). Dorsal hippocampus E2 infusion activates pyramidal neurons of the medial prefrontal cortex and the spinogenesis of CA1, which is depending on extracellular signal-regulated kinase (ERK) and the mammalian target of rapamycin (mTOR) (Tuscher et al., 2016). Using cortical neuronal cultures from rat embryos, Srivastava et al. demonstrated that the recruitment of the synaptic proteins PSD-95 and Nlg-1 as well as the NMDA receptor subunit GluN1 to newly formed spines is concurrent with E2-induced spinogenesis, which indicates that new spines form functional synapses (Sellers et al., 2015). They also reported that E2 activated ERK1/2 and Akt, which were required for

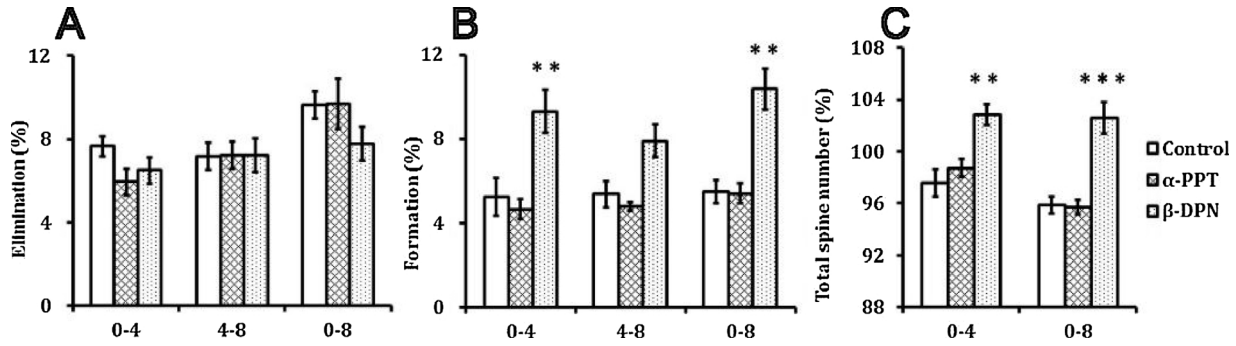

Fig. 4. Effects of α-PPT and β-DPN on spine turnover and density in the intact sensory cortex of female OVX mice (statistical analysis by Student's t-test). **(A)** Percentage of spine elimination during days 0–4 in control (n = 7), α-PPT (n = 8), and β-DPN (n = 9) mice (α-PPT, $P > 0.05$; β-DPN, $P > 0.05$), during days 4–8 in control (n = 7), α-PPT (n = 6), and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, $P > 0.05$), and at days 0–8 in control (n = 7), α-PPT (n = 6), and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, $P > 0.05$). **(B)** Percentage of spine formation at days 0–4 in control (n = 7), α-PPT (n = 8), and β-DPN (n = 9) mice (α-PPT, $P > 0.05$; β-DPN, **$P < 0.01$; *vs.* control), at days 4–8 in control (n = 7), α-PPT (n = 6), and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, $P > 0.05$; *vs.* control), and at days 0–8 in control (n = 7), α-PPT (n = 6) and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, **$P < 0.01$; *vs.* control). **(C)** Percentage of spine density at days 4–8 in control (n = 7), α-PPT (n = 6), and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, **$P < 0.01$; *vs.* control), at days 4–8 in control (n = 7), α-PPT (n = 6), and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, $P > 0.05$; *vs.* control), and at days 0–8 in control (n = 7), α-PPT (n = 6), and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, ***$P < 0.001$; *vs.* control).

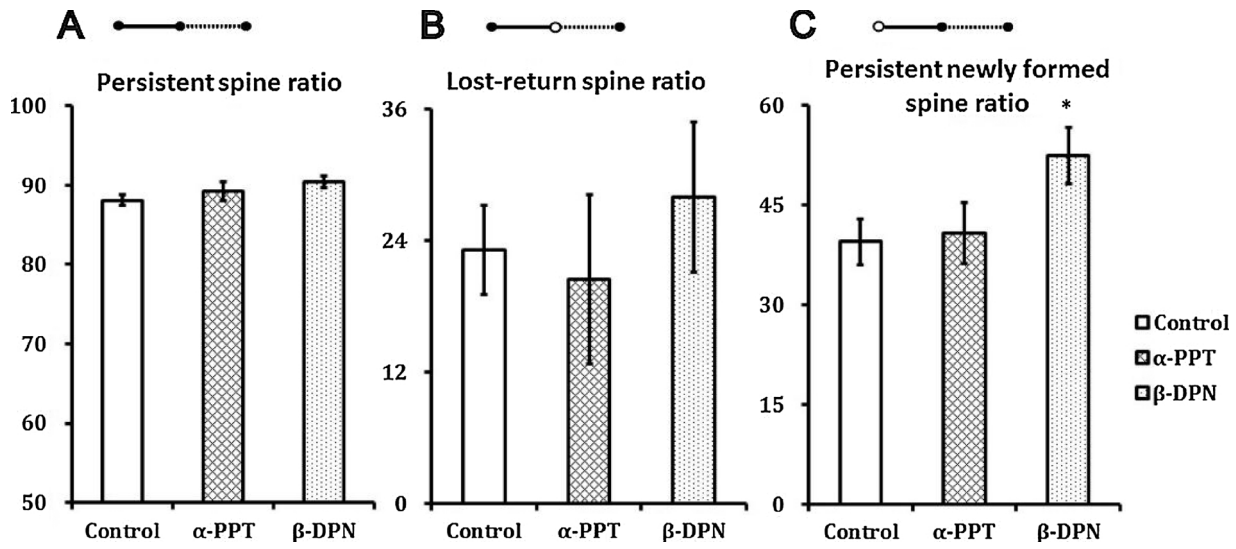

Fig. 5. Regulation of α-PPT and β-DPN on the fates of different spines in cortical neurons of female OVX mice (statistical analysis by Student's t-test). (A) Persistent spine ratio corresponding to the fate of pre-existing spines in control (n = 7), α-PPT (n = 6), and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, $P > 0.05$; *vs.* control). (B) Lost-return spine ratio corresponding to the fate of lost spines in control (n = 7), α-PPT (n = 6), and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, $P > 0.05$; *vs.* control). (C) Persistent newly formed spine ratio corresponding to the fate of newly formed spines in control (n = 7), α-PPT (n = 6), and β-DPN (n = 8) mice (α-PPT, $P > 0.05$; β-DPN, $^*P < 0.05$; *vs.* control).

E2-induced spinogenesis and mTOR kinase pathways, which contributed to E2-mediated cognitive enhancements. In addition, activation of ERβ, but not ERα, was sufficient to increase the density of PSD-95-containing spines. Taken together, these studies suggest that, through modulation of diverse molecular pathways by ERβ, E2 is capable to induce synaptogenesis as well as spinogenesis; however, these studies do not provide sufficient morphological evidence. In our work, we directly demonstrated through *in vivo* tracing the fate of individual spines that E2-induced spines are selectively stabilized and retained for at least 4 d, which should be sufficient for these spines to become functional synapses (Knott et al., 2006). Furthermore, we showed that ERβ plays a critical role in the modulation of E2 on both spinogenesis and synaptogenesis in the sensory cortex.

In our study, we examined the effects of E2 and ER agonists on spine plasticity in the sensory cortex. It is well established that the sensory cortex plays an important role in reception, storage, and transition of sensory information. Various sensory inputs, such as input from whiskers (Fox, 2002; Erzurumlu and Gaspar, 2012), are transmitted to the sensory cortex for further processing. Sensory experience can modify synaptic connectivity, which is considered as a basis for information storage in the sensory cortex (Yang et al., 2009; Zuo et al., 2005; Matsuzaki et al., 2004; Nicoll and Malenka, 1999; Shi et al., 1999). For instance, long-term sensory deprivation reduces the rate of spine elimination, and restoring sensory experience from deprivation accelerates spine elimination in the intact sensory cortex. *In vivo* studies also showed that novel sensory experiences induce the formation and selective stabilization of new synapses (Yang et al., 2009). These data suggest that long-lasting synaptic reorganization, including both experience-based pruning of pre-existing connections and novel experience-induced newly formed and then stabilized connections, serves as an important mechanism in learning and memory. The sensory cortex is an ideal model for *in vivo* investigating the mechanism of synaptic plasticity for structurally integrating new information into the neuronal circuitry at the level of individual synapses. In our study, we also chose the sensory cortex to investigate the effects of E2 on synaptic plasticity in the cerebral cortex because it is a large region and more accessible for the open-skull procedure compared with other areas of the cortex. Furthermore, the sensory input to the sensory cortex can be easily manipulated, which is convenient for understanding if and how E2 influences re-wiring of neural circuits in the cortex during novel experiences in future work.

An increasing body of evidence suggests that spinogenesis and remodeling in the cortex, such as the sensory, motor, and visual cortex, are involved in learning and memory (Matus, 2005; Yang et al., 2009; Xu et al., 2009; Hofer et al., 2009). Spinogenesis caused by estrogen treatment via the ERβ pathway is very similar to that induced by learning or sensory experience related with the cortex. Learning and novel sensory experiences have been reported to lead to spine formation (Yang et al., 2009; Xu et al., 2009) and the increase in the survival rate of newly formed spines, both of which correlates with behavioral improvement (Yang et al., 2009; Xu et al., 2009). Our study showed that activation of ERβ promoted either growth or stability of new dendritic spines. Based on our results, we propose that the ERβ pathway may play a positive effect on improving brain cognition correlated with the sensory cortex. In addition, accumulated clinical evidence suggests that estrogen exposure decreases the risk and delays onset and progression of a variety of neuropsychiatric disorders, including Parkinson's disease, depression, and anxiety (Garcia-Segura et al., 2001). Specially, clinical patients with major depression are accompanied by atrophy of somatosensory cortex volume and sensory processing disorders (Schmaal et al., 2017; Serafini et al., 2017). Animal studies showed that stress experience lead to obvious loss of dendritic spines in the mouse barrel cortex, which closely associates with deficits in a whisker-dependent texture discrimination task learning (Schmaal et al., 2017; Serafini et al., 2017). It was reported that selective activation of ERβ has anti-depressive-like effects in many cognitive tests as well (Walf and Frye, 2008). In view of both the declining of memory (Guo et al., 2015; Penzes et al., 2011) and the loss of synaptic connectivity are common hallmarks of the above diseases, the increase of stable neural connectivity triggered by E2 through enhancing the new spine formation and retaining reverses the loss of connectivity in these diseases to a certain extent, which provides a new clue for understanding the neurological mechanism of E2 for adjunctive therapy of the above diseases at the neural circuit level.

Our future studies will focus on investigating whether E2 modulates the sensory impulse stimulating adaptive learning, elucidating the underlying mechanism of the synapse dynamics in this process, and determining which estrogen receptor pathway is involved.

4. Methods

4.1. Animals

In the present study, we chose young adult (8–12 weeks of age) female *Thy1*-GFP-M transgenic mice, which were sparsely labelled with green fluorescent protein (GFP) at the layer V cortical pyramidal neurons as subjects. Mice were purchased from the Jackson Laboratory (JAX, Maine, USA) and housed and bred in the specific pathogen-free (SPF) animal facilities at Wuhan National Laboratory for Optoelectronics. Mice were group-housed until open-skull surgery in a quiet room (22–23 °C) with a 12 h light/dark cycle. After surgery, the mice were singly housed. Food and water were provided ad libitum. All procedures were carried out in compliance with protocols approved by the Hubei Provincial Animal Care and Use Committee and the experimental guidelines of the Animal Experimentation Ethics Committee of Huazhong University of Science and Technology, China.

4.2. Ovariectomy

Female mice were gonadectomized at the age of 9–11 weeks. Sterilized all of the surgical instruments. Mice were anesthetized using a ketamine cocktail (17 mg/mL ketamine and 1.7 mg/mL xylazine) via intraperitoneal injection. The dorsum was rubbed with 75% ethanol surgical scrub, and a 2 × 3 cm area was shaved with a sterile blade near the hump of the animal. Cut the flank skin layer to make a horizontal, 3 cm incision with Adson forceps. Then bilateral small muscle incisions were made to pull out and cut the ovaries which surrounded by a variable amount of fat from the oviduct. Muscle and skin were sutured with suture thread, which was absorbed a week post-surgery.

4.3. Open-skull surgical procedures

The skull of young, adult, female mice was opened and the mice were implanted with an elliptical cover-glass, as described previously (Guo et al., 2015; Shu and Xu, 2017). Briefly, we anesthetized mice with a ketamine cocktail containing 17 mg/mL ketamine and 1.7 mg/mL xylazine via intraperitoneal injection. Then, we stabilized the head of the animal in a stereotaxic equipment. After removing skin and periosteum, we used a high-speed drill to intermittently drill a circular groove in the skull above the primary sensory cortex. The island skull was removed by a lancet (without affecting the dura) and replaced with an elliptical cover-glass window. Cyanoacrylate and dental resin were applied to seal off the exterior, cover both exposed skull and wound edge, as well as to attach a titanium bar to the skull offside to the cranial window for stabilizing the head during the subsequent imaging sessions.

4.4. In vivo transcranial two-photon imaging

Our previous custom-built fixed device and two-photon microscope made consecutive, stable, high-resolution imaging on the apical dendrite of layer V pyramidal neurons possible (Guo et al., 2015; Shu and Xu, 2017). The commercial Olympus microscope (Olympus Corporation, Tokyo, Japan; FV1200, equipped with a Mai Tai Ti:sapphire laser) was repeatedly used to image the apical dendrites of layer V pyramidal neurons. The imaging location was within an area of 9–20 mm (Marinho et al., 2008) on the central point at 3.4 mm lateral from the midline and 1.1 mm posterior to the bregma (Yang et al., 2009). The emission wavelength was 925 nm with a low laser power that could abate phototoxicity. An Olympus water-immersion objective with a high numerical aperture (NA = 1.05) facilitated taking high-resolution images. In addition, we chose 0.7 μm as a suitable step size because the spine position can shift by up to ~0.3 μm in either direction along the axes of dendritic branches owing to morphology changes and/or tissue rotation (Grutzendler et al., 2002). The animal was returned to its original cage until the next imaging session and repeatedly anesthetized during each imaging phase. In order to relocate a certain image area at later time points, the pattern of the brain vasculature was taken with a CCD camera as a map, and the dendrites of interest were taken in the low-resolution image stack with a step size of 2.0 μm as landmarks. In conjunction with the vasculature map, the dendrites served as higher resolution landmarks for accurate relocation and alignment of the same region for later imaging.

4.5. Drug treatment

All mice subjected to ovariectomy were divided into several groups according to the hormone administration approach: experimental groups were treated with 17β-estradiol (E2), ERβ agonist (β-DPN), or ERα agonist (α-PPT). E2-release pellets (1.5 mg/pellet) were purchased from Innovative Research of America (IRA, Florida, USA), and implanted after the onset of imaging on the lateral side of the mouse neck between ear and shoulder. A solution of β-DPN/α-PPT in sesame oil (all compounds purchased from Sigma Aldrich, St. Louis, USA) was subcutaneously injected at the dose of 0.1 mL/10 g with a concentration of 25 μg/0.1 mL once a day for eight days. The doses of PPT and DPN on this subject specifically referenced to the previous task-effective reports (Phan et al., 2011).

4.6. Statistical analysis

Experimental data were analyzed using the Student's *t*-test. The procedure for quantifying spine dynamics has been described before (Guo et al., 2015; Penzes et al., 2011). It is worth mentioning that we accurately relocated the same randomly chosen and previously imaged dendritic branch (~10–20 μm in length) from three-dimensional image stacks taken with high-resolution. The dendritic spines quantified in our study were apical dendrites. Independent on their branch orders, the dendrites were taken at ~100 μm below the cortical surface. Images of the spine dynamics were analyzed manually on the raw image stacks using the Image J software blind to the experimental conditions. The number of mice in each experiment was at least four per group.

Ethical approval

All animal experiments were approved by the Hubei Provincial Animal Care and Use Committee and the experimental guidelines of the Animal Experimentation Ethics Committee of Huazhong University of Science and Technology, China.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors contributions statement

Shaofang Wang and Tonghui Xu designed research. Shaofang Wang and Jun Zhu performed the research. Shaofang Wang, Jun Zhu and Tonghui Xu analyzed data and wrote the paper. All authors read and approved the final manuscript.

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