Estradiol modulates the efficacy of synaptic inhibition by decreasing the dwell time of $GABA_A$ receptors at inhibitory synapses

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Estrogen plays a critical role in many physiological processes and exerts profound effects on behavior by regulating neuronal excitability. While estrogen has been established to exert effects on dendritic morphology and excitatory neurotransmission its role in regulating neuronal inhibition is poorly understood. Fast synaptic inhibition in the adult brain is mediated by specialized populations of γ-c a_A receptors ($GABA_A$Rs) that are selectively enriched at synapses, a process dependent upon their interaction with the inhibitory scaffold protein gephyrin. Here we have assessed the role that estradiol (E2) plays in regulating the dynamics of $GABA_A$Rs and stability of inhibitory synapses. Treatment of cultured cortical neurons with E2 reduced the accumulation of $GABA_A$Rs and gephyrin at inhibitory synapses. However, E2 exposure did not modify the expression of either the total or the plasma membrane $GABA_A$Rs or gephyrin. Mechanistically, single-particle tracking revealed that E2 treatment selectively reduced the dwell time and thereby decreased the confinement of $GABA_A$Rs at inhibitory synapses. Consistent with our cell biology measurements, we observed a significant reduction in amplitude of inhibitory synaptic currents in both cultured neurons and hippocampal slices exposed to E2, while their frequency was unaffected. Collectively, our results suggest that acute exposure of neurons to E2 leads to destabilization of $GABA_A$Rs and gephyrin at inhibitory synapses, leading to reductions in the efficacy of GABAergic inhibition via a postsynaptic mechanism.

estrogen | inhibition | synapse

Estrogens exert profound effects on neuronal excitability which are likely to underlie, for example, their role in seizure disorders and in regulating cognitive function (1, 2). Consistent with this, estrogen has been shown to potentiate excitatory neurotransmission, likely through effects on glutamate receptor trafficking (3–5). In contrast, their effects on inhibitory neurotransmission mediated through $GABA_A$ receptors ($GABA_A$Rs) remain relatively poorly described and mechanistically obscure.

$GABA_A$Rs are chloride-selective pentameric ligand-gated ion channels that are coassembled from a diverse array of subunits α (1–3), β (1–3), γ (1–3), δ, ε, and π with the majority of benzodiazepine-sensitive synaptic $GABA_A$Rs being composed of α (1–3), β (1–3), and γ2 subunits (6–8). The number of $GABA_A$Rs at inhibitory synapses is a critical determinant of the efficacy of phasic GABAergic inhibition, a process that is orchestrated by a family of receptor-associated proteins. Central to the accumulation of $GABA_A$Rs at inhibitory synapses is the multifunctional scaffold protein gephyrin, which is capable of oligomerization, forming a hexagonal lattice, in addition to binding both actin and microtubules (9). Gephyrin binds directly to conserved amino acid motifs within the intracellular loop domain of the $GABA_A$R α1–3 subunits, acting as a bridge to link these receptors to the cytoskeleton. Consistent with this concept, single-particle tracking (SPT) experiments have revealed that gephyrin selectively traps and reduces the mobility of $GABA_A$Rs at inhibitory synapses, thereby enriching their accumulation at these subcellular specializations (10–13).

Here we have examined the role that estrogen plays in regulating the cell surface dynamics of $GABA_A$Rs and stability of inhibitory synapses. Treatment of cultured cortical neurons with estradiol (E2) disrupts the clustering and reduces the confinement of $GABA_A$Rs at inhibitory synapses without altering either their total or plasma membrane protein expression. Additionally, we show that E2 drives a selective reduction in amplitude of inhibitory synaptic currents in both cultured cortical neurons and male hippocampal slices. This regulatory mechanism may have profound effects on the efficacy of neuronal inhibition and may contribute to the effects of estrogen on synaptic plasticity and disease pathology, including disorders with seizures and cognitive deficits.

Results

E2 Treatment Reduces the Number of Synaptic $GABA_A$Rs and Gephyrin in Cultured Cortical Neurons. To initiate our study, we examined the effect of the stable estrogen analog E2 on the stability of inhibitory synapses in cultured cortical [~24 d in vitro (DIV)]

Significance

Our knowledge of how estrogen signaling can influence inhibitory synaptic transmission is rudimentary and is addressed here. Collectively, our data suggest that estrogen modulates the dynamics of surface $GABA_A$ receptors and hence efficacy of synaptic inhibition, via a postsynaptic mechanism that relies on disrupting the postsynaptic scaffold. This regulatory mechanism may have profound effects on the efficacy of neuronal inhibition and therefore synaptic plasticity and thus play a role in the pathophysiology of estrogen-related seizure and cognitive disorders.

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neurons. Cultures were treated with E2 (10 nM) for 2 h and then stained with antibodies against an extracellular epitope for the α2 subunit and gephyrin and analyzed by confocal microscopy. Surface α2 subunit-containing $GABA_A$Rs were chosen, as they are largely restricted to gephyrin-enriched inhibitory postsynaptic sites and are major mediators of phasic inhibition in the brain (14, 15). For the analysis, a cluster is considered synaptic if it is colocalized with the inhibitory scaffolding protein gephyrin. The number of synaptic α2 clusters was compared per 30 μm of dendrite. E2 significantly decreased the number of synaptic α2 puncta (Fig. 1 *A* and *B*; control = 9.7 ± 0.5 and E2 = 3.74 ± 0.32 clusters per 30 μm; $P < 0.001$, unpaired *t* test, $n = 25$–26 cells). We also compared the total fluorescence intensity of remaining clusters, a combined measure of the average intensity and area of an individual cluster, by normalizing this value to that seen in vehicle-treated neurons. E2 treatment significantly decreased the intensity of remaining synaptic α2 puncta to 78.6 ± 2.6% of control (Fig. 1*B*; $P < 0.001$; unpaired *t* test, $n = 25$ cells).

To confirm our results with the α2 subunit we also examined the effects of E2 on the synaptic accumulation of the γ2 subunit, which plays an essential role in facilitating the targeting of $GABA_A$Rs to inhibitory synapses (16). E2 treatment significantly reduced the number of synaptic γ2 puncta (Fig. 1*C*; control = 14.3 ± 0.57 and E2 = 5.71 ± 0.35 clusters per 30 μm, $P < 0.001$, unpaired *t* test, 25 cells). The average fluorescence intensity of the remaining synaptic γ2 puncta was also significantly reduced to 78.2 ± 2.5% of control (Fig. 1*D*; $P < 0.001$; unpaired *t* test, $n = 25$). We also assessed the effects of E2 on inhibitory synapses containing the α1 subunit, the most abundant receptor α-subunit isoform expressed in the adult brain (17). Compared with control, E2 also reduced the number of synaptic α1 puncta (Fig. S1*A*).

Estrogen signals via a range of receptors including the canonical nuclear hormone receptors estrogen receptor α (ERα) and β (ERβ) and the G protein-coupled ER (GPER/GPR30) (18, 19). To begin to address which ERs mediate the effects of E2 on inhibitory synapses, we exposed cortical cultures to the ERα agonist [4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol] (PPT) (10 nM) or the ERβ agonist WAY-200070 [7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol] (070) (100 nM) for 2 h. Both of these agents induced reductions in the number of inhibitory synapses similar to those seen with E2 (Fig. S2). Compared with control (DMSO), both 070 and PPT significantly decreased the number of synaptic α2 puncta (Fig. S2*B*; control = 9.1 ± 0.4, 070 = 4.2 ± 0.32, PPT = 5.6 ± 0.38 clusters per 30 μm; $P < 0.001$, control vs. 070 and PPT, one-way ANOVA with Bonferroni's post hoc test, $n = 29$–32 cells). We also compared the total fluorescence intensity of remaining clusters, a combined measure of the average intensity and area of an individual cluster, by normalizing this value to that seen in vehicle-treated neurons. The 070 and PPT treatment significantly decreased the intensity of remaining synaptic α2 puncta (070, 82 ± 2.9% of control and PPT, 79.8 ± 1.8% of control, Fig. S2*B*; $P < 0.001$; one-way ANOVA with Bonferroni's post hoc test, $n = 29$–32 cells).

Finally, we assessed if ERs are expressed on the plasma membrane of neurons and whether they are found in the vicinity of inhibitory synapses. Due to the paucity of reliable, high-affinity antibodies to detect estrogen receptors, we exposed neurons to fluorescently labeled E2 coupled to BSA (FITC-E2) as a means of labeling cell surface populations of ERs (20). FITC-E2 labeled the plasma membrane population of ERs of live neurons, which was blocked by preincubation with E2, demonstrating the specificity of this staining (Fig. S3*A*). Significantly, FITC-E2 labeling was found within the same neuron and close to the puncta containing $GABA_A$Rs, suggesting E2 can potentially influence the clustering of $GABA_A$Rs via a local signaling mechanism (Fig. S3*B*).

Collectively, these results reveal that acute exposure of cortical neurons to E2 disrupts the clusters of the $GABA_A$Rs containing α1, α2, and γ2 subunits and gephyrin at the inhibitory synapses and the effects of E2 at these structures are mediated in part by ERα and ERβ.

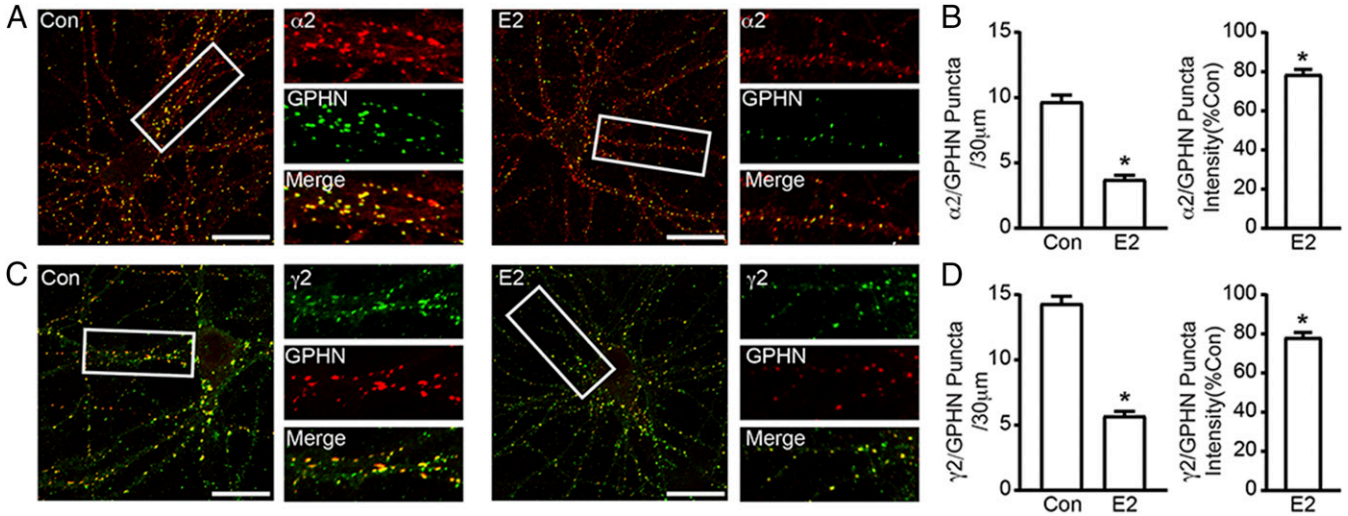

Fig. 1. E2 decreases the number and size of synaptic $GABA_A$Rs and gephyrin in cultured neurons. Cortical neurons (DIV ~24) were treated with E2 (10 nM) or DMSO (Con) for 2 h. Neurons were fixed and stained with anti-(α2, γ2) subunit antibody and following permeabilization, with an anti-gephyrin (GPHN) antibody. Large panels are the merged image of the maximum intensity projection of a representative confocal image. Right-hand panels represent enlargements of the boxed areas consisting of individual and merged channels. (Scale bars: 20 μm.) (*A*) E2 reduced the clustering of synaptic α2-$GABA_A$Rs. Images showing the clustering of α2 (red) and gephyrin (green), control (*Left*), E2 (*Right*). (*B*) The number of α2/gephyrin clusters per 30 μm was compared between treatments in the right-hand panel. In the left-hand panel cluster intensity was compared by normalized values to those seen in control (100%). In both panels asterisks indicate significantly different from control, $P < 0.001$ ($n = 25$ cells). (*C*) E2 reduced the synaptic clustering of γ2-$GABA_A$Rs. Images showing the clustering of γ2 (green) and GPHN (red), control (*Left*) and E2 (*Right*). (*D*) The number of γ2/gephyrin clusters per 30 μm was compared between treatments in the right-hand panel. In the left-hand panel cluster intensity was compared by normalized values to those seen in control (100%). In both panels * indicates significantly different from control, $P < 0.001$ ($n = 25$ cells). All data are presented as mean ± SEM.

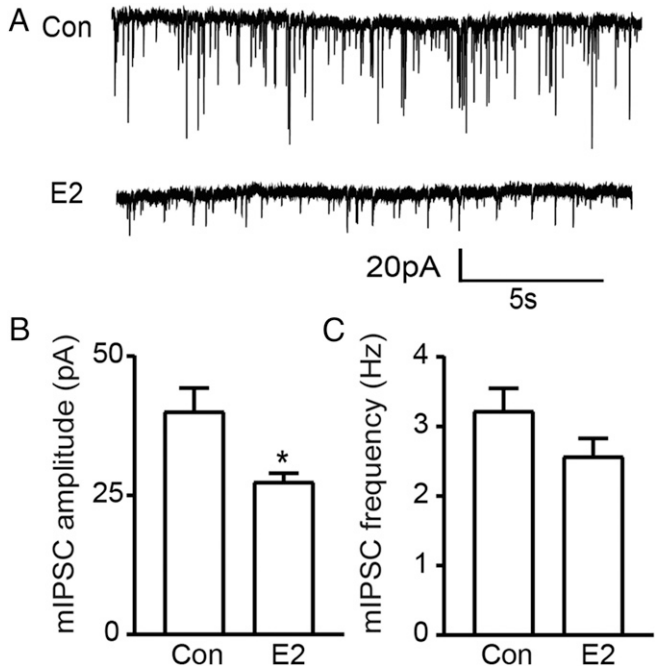

Fig. 2. E2 selectively reduces the amplitude on mIPSC in cultured cortical neurons. (*A*) Sample traces are shown of mIPSCs recorded from neurons (DIV ~24 d) either treated with DMSO (Con) or E2 (10 nM) for 2 h. (*B*) The bar graph shows the decrease in the average mIPSC amplitude per cell (unpaired *t*-test; $P = 0.01$, $n = 14$–16 cells) (*C*) The graph shows no significant changes in the average mIPSC frequency per cell ($P = 0.14$; *t* test, $n = 14$–16 cells). All data are presented as mean ± SEM.

E2 Selectively Reduces the Amplitude of Miniature Inhibitory Synaptic Currents in Cultured Cortical Neurons. Given the results of our imaging studies, we tested if E2 leads to changes in the properties of miniature inhibitory synaptic currents (mIPSCs) in cultured cortical neurons (DIV ~24). These events were isolated using the glutamate receptor antagonists CNQX/AP5 and in the presence of TTX. Exposure to E2 (10 nM) ($n = 14$–16 cells) decreased the size of postsynaptic GABA currents (Fig. 2*A*). A cumulative amplitude histogram revealed a leftward shift toward smaller mIPSC amplitudes in response to E2 treatment (Fig. S4*A*). Accordingly, the average mIPSC amplitude was significantly reduced upon exposure to E2 (Fig. 2*B*; control = 40.1 ± 4.2 pA, E2 = 27.4 ± 1.6 pA, $P = 0.01$, unpaired *t* test, $n = 14$–16). E2 exposure did not modify mIPSC rise time (control $\tau_{rise} = 4.99 \pm 0.2$ ms, E2 $\tau_{rise} = 4.90 \pm 0.16$ ms, $P = 0.72$, unpaired *t* test) or decay (control $\tau_{decay} = 21.02 \pm 0.6$ ms, E2 $\tau_{decay} = 20.40 \pm 0.6$ ms, $P = 0.48$, unpaired *t* test, $n = 14$–16). Likewise, E2 did not modify mIPSC frequency (Fig. 2*C*; control = 3.22 ± 0.33 Hz, E2 = 2.57 ± 0.26 Hz, $P = 0.14$, unpaired *t* test, $n = 14$–16).

Collectively, our electrophysiological measurements suggest that E2 acts to selectively reduce the amplitude of mIPSCs, an effect that is consistent with its ability to reduce the size and number of synaptic $GABA_A$Rs (Fig. 1).

E2 Does Not Modify the Cell Surface or Total Protein Expression of $GABA_A$Rs or Gephyrin. The number of $GABA_A$Rs on the neuronal membrane has profound effects on the efficacy of GABAergic inhibition, a process that is critically dependent upon regulated receptor exo- and endocytosis (21, 22). Therefore, we assessed if exposure to E2 modifies the cell surface expression of $GABA_A$Rs subunits. To isolate surface $GABA_A$Rs, cultured cortical neurons were exposed to NHS-Biotin and after purification on streptavidin beads; cell surface and total fractions were immunoblotted with antibodies against the α1 and γ2 subunits. This revealed that, compared with control, exposure to E2 did not significantly alter the cell surface stability of α1 or γ2 subunits (Fig. 3*A*; α1: 98 ± 10.96%, $P = 0.87$ and γ2: 105.8 ± 1.8%, $P = 0.08$ of control, respectively, unpaired *t* test, $n = 3$). Significantly, the surface fractions were free of the cytosolic protein GAPDH, verifying the integrity of our biotinylation procedure (Fig. 3*A*). Likewise, E2 did not modify the total expression of $GABA_A$R subunits (Fig. 3*B*; α1 = 102.2 ± 8%, $P = 0.81$ and γ2: 101.4 ± 5.7%, $P = 0.83$ of control, respectively, unpaired *t* test, $n = 3$). Moreover, the total expression level of gephyrin was comparable in neurons treated with E2, (Fig. 3*B*; 107.9 ± 4.2% of control, $P = 0.13$, unpaired *t* test, $n = 5$).

Next, we assessed if E2 treatment modifies the association of gephyrin with $GABA_A$Rs. We subjected neuronal lysates to immunoprecipitation (IP) with an antibody against the α1 subunit or mouse IgG as a control (10). Precipitated material was then immunoblotted with gephyrin and α1-subunit antibodies, and the ratio of gephyrin immunoreactivity was compared between control and E2 treatment. This approach revealed that E2 did not significantly alter the association of $GABA_A$Rs with gephyrin (Fig. 3*C*; 121.8 ± 10.9% of control, $P = 0.18$, unpaired *t* test, $n = 3$).

Therefore, E2 does not act to modify either the total or cell-surface levels of synaptic $GABA_A$Rs or the stability of the inhibitory

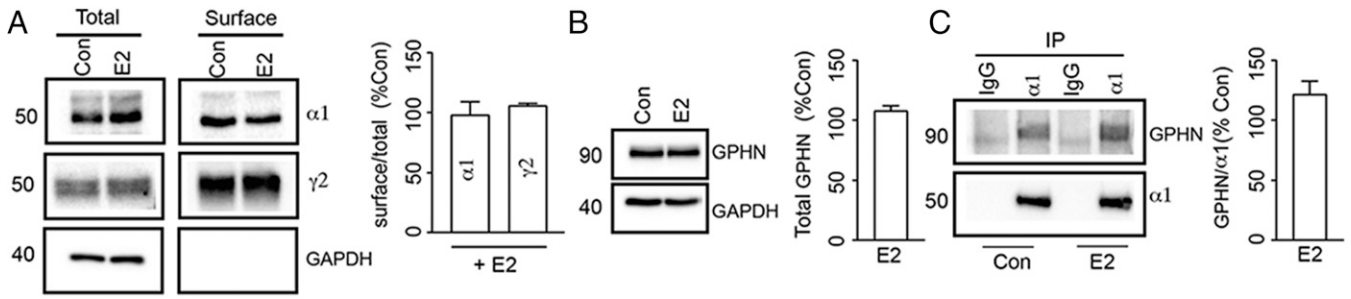

Fig. 3. E2 does not affect the total or surface level expression of the $GABA_A$R or the stability of the $GABA_A$R–gephyrin complex. Cortical neurons (DIV ~24) were treated with E2 (10 nM) or DMSO (Con) for 2 h and subject to immunoblotting after biotinylation or co-IP. Indicated molecular weights are in kilodaltons. (*A*) Blots showing the total (*Left*) and the surface (*Right*) expression of the $GABA_A$R subunits, immunoblotted with anti-α1 or -γ2 $GABA_A$R subunit and anti-GAPDH antibodies. The bar graph shows the surface/total levels of each subunit that was normalized to the control (100%). ($P > 0.05$, not significant, unpaired *t* test, $n = 3$.) GAPDH serves as loading control. (*B*) Blots showing the total expression of gephyrin. No significant change was observed after E2 treatment ($P = 0.13$, $n = 5$). GAPDH serves as loading control. (*C*) Detergent-solubilized cell extracts were subject to IP with IgG or α1 antibodies. Precipitated material was then immunoblotted with anti-α1 and anti-GPHN antibodies. The ratio of GPHN/α1 immunoreactivity was determined and normalized to the control (100%). ($P = 0.18$, $n = 3$.) All data are presented as mean ± SEM.

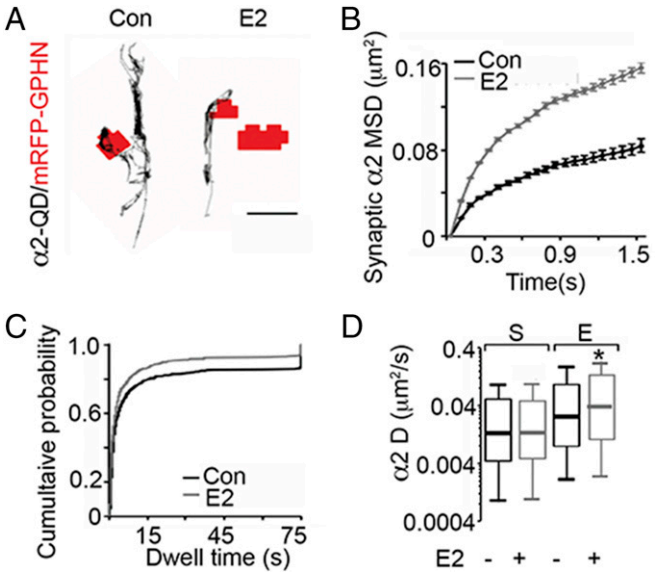

Fig. 4. E2 decreases the dwell time of $GABA_A$Rs at inhibitory synapses. Cortical neurons (DIV ~24) from mRFP-gephyrin transgenic mice were treated with E2 (10 nM) or DMSO (Con) for 2 h. (*A*) Representative reconstructed traces of QD-α2$GABA_A$Rs are shown (black). In both cases, synaptic membrane areas are indicated by the endogenous fluorescence of mRFP-gephyrin, segmented in red domains. (Scale bar: 1 μm.) (*B*) The graph shows the change in the MSD (square micrometers) over time of the endogenous QD-α2$GABA_A$Rs within synaptic membrane compartments, control (black) and E2 (gray). E2 treatment resulted in the increased explored area over time at the synaptic sites. (*C*) The graph shows the cumulative distribution of the dwell times of QD-α2$GABA_A$Rs on the mRFP-gephyrin, control (black) and E2 (gray). The residence time of endogenous synaptic α2-$GABA_A$Rs is decreased after E2 treatment. (*D*) Distribution of QD–α2$GABA_A$R complexes diffusion coefficients (D, square micrometers per second) in synaptic (S, left) and extrasynaptic (E, right) membrane compartments. After E2 exposure (gray), α2$GABA_A$Rs exhibit increased diffusion coefficient at extrasynaptic but not synaptic neuronal membranes. Box plots indicate the D value for (90, 75, 50, 25, and 10%) of the population.

scaffold gephyrin. Finally, exposure to E2 does not compromise the ability of gephyrin to bind $GABA_A$Rs.

SPT Reveals That ER-Mediated Signaling Reduces the Confinement of $GABA_A$Rs at Inhibitory Synapses. To assess the effects of E2 on the dynamics of inhibitory synapses we used a transgenic mouse strain that expresses gephyrin fused with a monomeric red fluorescent protein (mRFP-GPHN) (23). To track the movement of individual $GABA_A$Rs on neuronal membranes, cortical cultures were labeled with low concentrations of quantum dots (QDs) coupled to anti-α2 antibodies, allowing us to monitor the mobility of individual $GABA_A$Rs using the SPT technique (24). As an initial control, we measured the effects of E2 on the fluorescence intensity of the synaptic α2-$GABA_A$Rs and mRFP-GPHN. $GABA_A$Rs that are colocalized with gephyrin were considered to be synaptic and integrated fluorescence intensities, reflecting their relative accumulation, were quantified. Consistent with our immunofluorescence experiments with rat cortical culture, exposure to E2 reduced the intensity of α2-$GABA_A$Rs and mRFP-GPHN fluorescence in neurons (Fig. S5; α2: 75.2 ± 3.2% and mRFP-GPHN: 79.2 ± 2.6% of control $P < 0.0001$, unpaired *t* test, $n = 19$–20 cells).

The lateral diffusion of neurotransmitter receptors within cell membrane hinders its stability at the synapses. Therefore, we examined the potential effect of E2 on the lateral mobility of $GABA_A$Rs and monitored the dynamic behavior of QD-labeled endogenous α2-$GABA_A$Rs on neuronal membranes (Fig. 4*A*). To discriminate between synaptic and extrasynaptic receptor dynamics, only mRFP-GPHN–positive membrane compartments were defined as synaptic (25). At synapses, E2 enhanced the mean square displacement (MSD) of QD labeled-α2-$GABA_A$Rs, suggesting a large decrease of their confinement at synaptic sites (Fig. 4*B*). A reduced dwell time of α2-$GABA_A$Rs at inhibitory synapses was also observed, suggesting an enhanced dispersal and a decreased stability of $GABA_A$Rs within synapses [Fig. 4*C*; $P = 0.0075$, Kolmogorov–Smirnov (KS) test, control, $n = 309$ and E2, $n = 285$ synaptic trajectories]. Accordingly, the percentage of trajectories stabilized at synapses was decreased from 13.4 ± 2.4 to 7.48 ± 1.6% after E2 exposure ($P = 0.035$, unpaired *t* test, $n = 3$). The diffusion coefficient distribution of α2-$GABA_A$Rs was not modified, suggesting that their diffusion properties were not affected at synaptic membrane compartments. In contrast to this, a modest but significant increase in the diffusion coefficient of α2-$GABA_A$Rs located at extrasynaptic sites was measured upon exposure to (Fig. 4*D*; E2 $P = 2.94\text{E-}05$, KS test, $n = 1{,}188$, and 1,174 extrasynaptic trajectories for control and E2, respectively).

Thus, our results using SPT suggest that E2 acts to decrease the dwell time of $GABA_A$Rs at synaptic sites, which at a steady state accounts for a reduced accumulation of $GABA_A$Rs at synapses.

E2 Modifies the Efficacy of GABAergic Inhibition in Hippocampal Slices. To examine the significance of our findings using cultured cortical neurons we assessed the effects of E2 on the properties of spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from CA1 pyramidal neurons in hippocampal slices prepared from male (~3 mo old) C57/BL6 mice. Exposure of slices to E2 (10 nM) for 2 h significantly reduced the amplitude of sIPSCs in CA1 neurons (Fig. 5*A*). A cumulative amplitude histogram revealed a leftward shift toward smaller sIPSC amplitudes in response to E2 treatment (Fig. S4*B*), Likewise, the average sIPSC amplitude was significantly decreased upon E2 treatment (Fig. 5*C*; control: 46.1 ± 7.7 pA, E2: 29.4 ± 2.7 pA, $n = 11$–13 cells, three mice per experimental group, $P = 0.04$, unpaired *t* test). However, we have not seen any significant changes in their frequencies (Fig. 5*D*; control: 10.1 ± 2.1 Hz, E2: 7.3 ± 1.1 Hz, $n = 11$–13 cells, three mice per experimental group, $P = 0.21$, unpaired *t* test). Also, exposure to E2 did not have

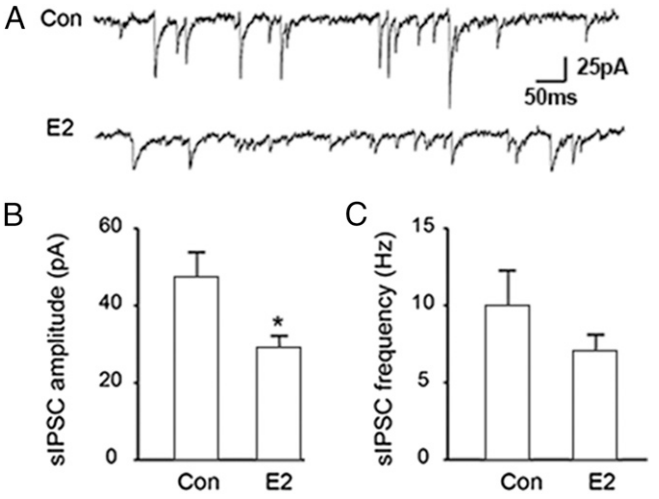

Fig. 5. E2 decreases the amplitude of sIPSC in male hippocampal slices without affecting frequencies. (*A*) Representative traces of sIPSCs recorded in CA1 pyramidal neurons from slices from male mice treated with vehicle (Con) or E2 (10 nM). (*B*) The average amplitude of sIPSCs is decreased following E2 treatment compared with vehicle ($P = 0.04$, unpaired *t* test, $n = 11$–13 cells). (*C*) The bar graph shows no significant changes in average frequencies of sIPSCs in either group ($P = 0.21$, unpaired *t* test, $n = 11$–13 cells).

any significant effect on the sIPSC decay (control: 7.4 ± 0.8 and E2: 7.0 ± 0.5 ms).

Collectively, these results further suggest a critical role for a postsynaptic mechanism in mediating the effects of E2 on GABAergic inhibition.

Discussion

Estrogen plays a key role in regulating neuronal activity and animal behavior and alterations in estrogen signaling are linked to a range of neurological and psychiatric conditions (26, 27). Estrogen regulation of excitatory neurotransmission has been heavily investigated (4, 5, 28) but how estrogen shapes inhibitory synaptic transmission is still poorly understood. It has been shown previously that E2 can suppress inhibitory synaptic transmission in young adult rat hippocampal slices—intriguingly, an effect only seen in females. Furthermore, it was shown to be mediated primarily via a presynaptic mechanism involving mGluR1 regulation of endocannabinoid signaling and subsequent regulation of GABA release from the presynaptic interneuron, which is engaged preferentially in females (29–31). However, to date, there have been no detailed studies to define the role that E2 plays in determining the dynamics of $GABA_A$Rs at inhibitory synapses.

To address this issue, we examined the effects of E2 on the number of inhibitory synapses in cultured cortical neurons. Exposure to E2 reduced the number of gephryin-positive inhibitory synapses. Parallel electrophysiological studies revealed that E2 induced a reduction in the amplitudes of mIPSCs, without changing their frequency, consistent with the removal of $GABA_A$Rs from synaptic sites. Collectively, these results suggest that E2 acts to reduce the stability of inhibitory synapses and/or the number of functional $GABA_A$Rs at these structures via a postsynaptic mechanism.

The efficacy of GABAergic inhibition and the maintenance of inhibitory synapses are in part determined by the plasma membrane stability of $GABA_A$Rs. This process is dependent upon the rates of receptor exo- and endocytosis, which is in turn subject to dynamic modulation by neuronal activity (22, 32). Therefore, we examined the effects of E2 on the accumulation of $GABA_A$Rs on the plasma membrane. Our results revealed that E2 did not significantly modify either the total expression levels or the cell-surface accumulation of $GABA_A$Rs. The stability of synaptic $GABA_A$Rs clusters is largely dependent on the integrity of a gephyrin scaffold. Multiple studies have documented compromised synaptic clustering of $GABA_A$Rs (e.g., α2 and γ2), upon reduction of gephyrin (14, 16, 33). We have provided direct evidence that E2 does lead to the disruption of gephyrin clusters as shown by a reduction in the numbers and fluorescence intensities of individual puncta. Since we did not observe any significant change in the level of expression of gephyrin, a reduction in the fluorescence intensity is very likely due to the destabilization of the gephyrin scaffold upon E2 treatment.

To further evaluate the mechanism by which E2 modulates the stability of inhibitory synapses we examined the effects of E2 on the mobility of synaptic $GABA_A$Rs in real time. SPT data reveal that E2 selectively increased the MSD of synaptic $GABA_A$Rs and decreased their dwell time at these structures. We also noted a modest but significant increase in the diffusion coefficient of α2-$GABA_A$Rs located at extrasynaptic sites. This is likely due to the interaction of α2-$GABA_A$Rs with the scaffolding protein gephyrin outside of the synapse, as reported previously for glycine receptors, where it is estimated that 40% of receptor/gephyrin puncta are extrasynaptic (34). Therefore, our data clearly demonstrate that treatment with E2 resulted in reduced confinement of $GABA_A$Rs at synaptic sites.

Finally, we examined the effects of E2 on the efficacy of GABAergic inhibition in hippocampal slices from male mice. In CA1 neurons, E2 decreased sIPSC amplitude but without significant changes in frequency, which is consistent with our results using cultured cortical neurons. Previously, it has been reported that the suppression of amplitude of IPSCs in hippocampal slices was only seen in females due to a sex-specific endocannabinoid-dependent presynaptic mechanism (30). These apparent discrepancies might be due to methodological differences and/or difference in species used. However, our data strongly suggests that E2 can attenuate inhibitory synaptic transmission via a postsynaptic mechanism.

The precise mechanism by which E2 influences the stability of inhibitory synapses remains to be determined, but our results suggest that the effects of E2 are in part mediated via the activation of ERα and/or ERβ. In addition to modulating transcription, these receptors can exert a rapid nongenomic effect on cells by modulating MAPK signaling, GSK3β, and the activity of small GTPases (18–20). Interestingly, it has been shown previously that CaMKII, MAPK, or GSK3β can phosphorylate gephyrin in an activity-dependent manner and thereby regulate its stability (35–37). Therefore, it will be of interest to determine if E2 modulates gephyrin or $GABA_A$Rs subunit phosphorylation, a process that can have profound effects on the membrane trafficking and stability of these key components of inhibitory synapses.

In summary, our studies provide a molecular mechanism by which estrogen acts to reduce the efficacy of GABAergic inhibition by decreasing the stability of inhibitory synapses. Such modulation may have profound effects on the maintenance of neuronal excitation/inhibition balance and thus contribute to the enhancement of cognition and epilepsy in which excessive estrogen signaling is believed to be of significance.

Materials and Methods

Biochemical Measurements, Confocal Imaging, and Image Analysis. Rat primary cortical neurons (DIV ~24) were used throughout unless otherwise stated. See *SI Materials and Methods* for detailed biochemical methods, reagents, imaging, and analysis.

Electrophysiology. Detailed methods on the electrophysiological recordings from both cultured neurons and brain slices are described in *SI Materials and Methods*.

SPT Experiments. Methods of the SPT experiment and analysis have been previously described (24) and are outlined in detail in *SI Materials and Methods*.

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