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ORIGINAL ARTICLE

Estrogen protects against the detrimental effects of repeated stress on glutamatergic transmission and cognition

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Converging evidence suggests that females and males show different responses to stress; however, little is known about the mechanism underlying the sexually dimorphic effects of stress. In this study, we found that young female rats exposed to 1 week of repeated restraint stress show no negative effects on temporal order recognition memory (TORM), a cognitive process controlled by the prefrontal cortex (PFC), which was contrary to the impairment in TORM observed in stressed males. Concomitantly, normal glutamatergic transmission and glutamate receptor surface expression in PFC pyramidal neurons were found in repeatedly stressed females, in contrast to the significant reduction seen in stressed males. The detrimental effects of repeated stress on TORM and glutamate receptors were unmasked in stressed females when estrogen receptors were inhibited or knocked down in PFC, and were prevented in stressed males with the administration of estradiol. Blocking aromatase, the enzyme for the biosynthesis of estrogen, revealed the stress-induced glutamatergic deficits and memory impairment in females, and the level of aromatase was significantly higher in the PFC of females than in males. These results suggest that estrogen protects against the detrimental effects of repeated stress on glutamatergic transmission and PFC-dependent cognition, which may underlie the stress resilience of females.

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INTRODUCTION

It is known that corticosteroid stress hormones serve as an important regulator of cognitive and emotional processes.[1–3] Stress may either be deleterious or beneficial depending on the timing and intensity of stressors and individual responses: for example, short-term (acute) stressors usually elicit adaptive changes, whereas long-term (chronic) stress often results in maladaptive effects.[3] However, this pattern of stress responses appears to apply to only males. Studies using female rodents have obtained different conclusions concerning the impact of chronic stress on the central nervous system function.[4] For example, in male rats, restraint stress (RS; 6 h per day, 21 days) impairs performance on a variety of spatial memory tasks including radial arm maze,[5] object placement,[6,7] Y-maze[8] and water maze,[9] and a nonspatial, recognition memory.[6,7] In contrast, females exposed to the same stress paradigm show enhanced cognition and memory in almost all of these tasks.[7,9–15] These animal studies suggest that males are significantly more vulnerable to chronic stress, at least in terms of the measured cognitive behaviors, whereas females are far more resilient.[16]

The mechanisms for sex differences in the neurocognitive response to chronic stress are largely unknown. Because estrogen levels are different between males and females, and estrogen has neuroprotective properties in a number of *in vitro* and *in vivo* neural systems including those involved in cognitive functions,[17,18] it is conceivable that the interactive effects of this gonadal hormone with stress hormones might be important.[19] Consistently, delivery of a chimeric receptor, which blocks glucocorticoid receptor (GR) translocation and converts the glucocorticoid signal into a genomic effect of the estrogen receptor (ER), significantly reduces hippocampal lesion after neurological injury.[20] New evidence further demonstrates that estradiol can be produced within the brain, and might act at synapses, in addition to the nucleus, to alter neuronal excitability, synaptic transmission and plasticity.[21,22]

Stress has complex effects in the central nervous system, which is contributed by the temporal and spatial factors. Throughout the lifespan, the developing brain seems to be more sensitive to stressors.[23,24] Rats exposed to trauma as juveniles are more vulnerable to adverse effects of fear conditioning.[25] People exposed to early life stress have heightened vulnerability to anxiety and other mood disorders.[26] Among the multiple brain areas involved in cognition and emotion, the prefrontal cortex (PFC), a region controlling higher-level 'executive' functions, is a primary target of stress hormones.[3,27–29] Glutamate receptor-mediated synaptic transmission, which controls PFC network activity, is crucial for working memory subserved by PFC.[30,31] Our recent study has demonstrated that repeated stress negatively influences PFC-mediated cognitive processes by disturbing glutamatergic signaling in young male rats.[32] In this study, we provide electrophysiological, biochemical and behavioral evidence showing that repeated stress exerts differential effects on PFC glutamate receptors and PFC-dependent memory task in male vs female animals, in which the presence or absence of

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estrogen has an important role. These results provide potential molecular mechanisms underlying the sexually dimorphic stress responses in the PFC.

MATERIALS AND METHODS

Repeated stress paradigm

All experiments were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the State University of New York at Buffalo. Similar to our previous studies on stress,[33–35] we used prepubertal (~4 weeks old, juvenile–adolescent) Sprague–Dawley rats in this study, because it is a highly plastic period during which PFC undergoes critical postnatal development.[36] Rats were group-housed in cages with a light (0600–1800 hours)/dark (1800–0600 hours) cycle. For repeated RS, rats were placed in air-accessible cylinders for 2 h daily (1000–1200 hours) for 5–7 days (starting at p21–23). The container size was similar to the animal size, which made the animal almost immobile in the container. Experiments were performed 24 h after the last stressor exposure.

Behavioral testing

To test the impact of stress on cognitive functions, we measured the recognition memory task, a fundamental explicit memory process requiring judgments of the prior occurrence of stimuli based on the relative familiarity, special or recency information. Lesion studies have shown that the medial PFC has an obligatory role in the temporal order recognition memory (TORM),[37] so this behavioral task was selected and conducted as described.[32,37] All objects were cubes or polygons and composed of the same plastic, so that they could not readily be distinguished by olfactory cues. The height of the objects was ~7 cm, and all objects were affixed to a round platform (diameter: 61.4 cm) to prevent them from being displaced during a trial. Each rat was habituated two times on the platform for 5 min on the day of behavioral experiments. This TORM task comprised two sample phases and one test trial. In each sample phase, the animals were allowed to explore two identical objects for a total of 3 min. Exploration of an object was defined as directing the nose to the object at a distance of <2 cm and/or touching it with the nose. Turning around or sitting on the object was not considered exploratory behavior. Then, the animal was removed from the platform to its home cage. Different objects were used for sample phases I and II, with a 1-h delay between the sample phases. The test trial (3-min duration) was given 3 h after sample phase II. During the test trial, an object from sample phase I and an object from sample phase II were used. The positioning of the objects in space on the test trial was counterbalanced to avoid the impact of the objects' location. All behavioral experiments were performed at late afternoon and early evening in dim light. If temporal order memory is intact, the animals will spend more time exploring the object from sample I (i.e., the novel object presented less recently) compared with the object from sample II (i.e., the familiar object presented more recently). We calculated a discrimination ratio (DR), the proportion of time spent exploring the novel (less recent) object (i.e., the difference in time spent exploring the novel and familiar objects divided by the total time spent exploring both objects) during the test trial. This measure takes into account individual differences in the total amount of exploration time.

Electrophysiological recordings

Standard whole-cell voltage-clamp recordings was used to measure synaptic currents in rat layer V medial PFC pyramidal neurons in brain slices as we described previously.[32,33] Rats were killed after inhaling Halothane (Sigma, St Louis, MO, USA). Brains were immediately removed, iced and cut into 300-μm slices by a Vibratome (Leica VP1000S, Leica Microsystems Inc., Buffalo Grove, IL, USA). Slices were then incubated in artificial CSF (in mM: 130 NaCl, 26 $NaHCO_3$, 3 KCl, 5 $MgCl_2$, 1.25 NaH_2PO_4, 1 $CaCl_2$, 10 glucose, pH 7.4, 300 mOsm) for 1–6 h at room temperature (20–22 °C) bubbling with 95% O_2, 5% CO_2. PFC-containing slices were positioned in a perfusion chamber attached to the fixed stage of an upright microscope (Olympus, Center Valley, PA, USA) and submerged in continuously flowing oxygenated artificial CSF. Bicuculline (10 μM) and CNQX (25 μM) were added in *N*-methyl-D-aspartate receptor (NMDAR)-excitatory postsynaptic current (EPSC) recordings. Bicuculline and D-APV (25 μM) were added in α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid subtype glutamate receptor (AMPAR)-EPSC recordings. Patch electrodes contained internal solution (in mM): 130 Cs-methanesulfonate, 10 CsCl, 4 NaCl, 10 HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid), 1 $MgCl_2$, 5 ethylene glycol tetraacetic acid, 2.2 QX-314, 12 phosphocreatine, 5 MgATP, 0.2 Na_2GTP, 0.1 leupeptin, pH 7.2–7.3 and 265–70 mOsm. Cells were visualized with a ×40 water-immersion lens and illuminated with near infrared light and the image was detected with an infrared-sensitive CCD camera. A Multiclamp 700 A amplifier was used for these recordings. Tight seals (2–10 GΩ) from visualized neurons were obtained by applying negative pressure. With additional suction, the membrane was disrupted into the whole-cell configuration. Evoked EPSC were generated with a pulse from a stimulation isolation unit controlled by a S48 pulse generator (Astro Med, West Warwick, RI, USA). A bipolar stimulating electrode (FHC, Bowdoinham, ME, USA) was placed ~100 μm from the neuron under recording. Membrane potential was maintained at −70 mV for AMPAR-EPSC recordings. For NMDAR-EPSC, the cell (clamped at −70 mV) was depolarized to +60 mV for 3 s before stimulation to fully relieve the voltage-dependent Mg^{2+} block. Artificial CSF was modified to contain 1 mM $MgCl_2$ to record miniature EPSC in PFC slices (TTX-added).

To obtain the input–output responses, EPSC was elicited by a series of stimulation intensities (5–9 V) with the same duration of pulses (0.6 ms for NMDAR-EPSC; 0.06 ms for AMPAR-EPSC). In other experiments, synaptic currents evoked by the same stimulation intensity were recorded in individual neurons across groups with different manipulations. Data analyses were performed with Clampfit (Axon instruments, Molecular Devices, Sunnyvale, CA, USA) and Kaleidagraph (Albeck Software, Synergy Software, Reading, PA, USA). Synaptic currents were analyzed with Mini Analysis Program (Synaptosoft, Leonia, NJ, USA).

Biochemical measurement of surface and total proteins

To examine the surface expression of AMPAR and NMDAR, surface biotinylation was performed as described previously.[32,33] In brief, PFC slices were incubated with artificial CSF containing 1 mg ml^{-1} sulfo-*N*-hydroxysuccinimide-LC-Biotin (Pierce Chemical, Rockford, IL, USA) for 20 min on ice. The slices were then rinsed three times in Tris-buffered saline to quench the biotin reaction, followed by homogenization in modified radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% sodium dodecylsulfate, 0.5% deoxycholic acid, 50 mM $NaPO_4$, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 mg ml^{-1} leupeptin). The homogenates were centrifuged at 14 000 *g* for 15 min at 4 °C. Protein (15 μg) was removed to measure total protein. For surface protein, 150 μg of protein incubated with 100 μl of 50% Neutravidin Agarose (Pierce Chemical) for 2 h at 4 °C, and bound proteins were resuspended in sodium dodecylsulfate sample buffer and boiled. Quantitative western blots were performed on both total and biotinylated (surface) proteins using antibodies against GluR1 (1:500 (Millipore, Billerica, MA, USA), 05-855 or 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), sc-13152), GluR2 (1:500 (Millipore), MAB397), NR1 (1:500 (Millipore), 06-311

or 1:500 (Millipore), 05-432), NR2A (1:500 (Millipore), 07-632), NR2B (1:500 (Millipore), 06-600) and actin (1:1000 (Santa Cruz Biotechnology), sc-1616).

Small hairpin RNA lentiviral knockdown

To test the involvement of ER, small hairpin RNA (shRNA) was used for silencing rat ERα and rat ERβ gene expression. The shRNA oligonucleotide targeting rat ERα sequence (5′-GGCATG GAGCATCTCTACA-3′[38]) or rat ERβ sequence (5′-GATTCTGGA AATCTTTGACAT-3′; Open Biosystems, Lafayette, CO, USA) was inserted to the lentiviral vector pLKO.3G (Addgene, Cambridge, MA, USA), which contains an enhanced green fluorescent protein (eGFP) marker. For the production of lentiviral particles, a mixture containing the pLKO.3G shRNA plasmid (against ERα or ERβ), psPAX2 packaging plasmid and pMD2.G envelope plasmid (Addgene) was transfected to HEK-293FT cells using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). The transfection reagent was removed 12–15 h later, and cells were incubated in fresh Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum + penicillin/streptomycin) for 24 h. The media harvested from the cells, which contained lentiviral particles, were concentrated by centrifugation (2000 *g*, 20 min) with Amicon Ultra Centrifugal Filter (Ultracel-100K; Millipore). The concentrated virus was stored at − 80 °C. To test the knockdown effect, the ERα or ERβ shRNA lentivirus was added (1:200) to cortical cultures (7 days *in vitro*). At 7 days after infection, the neuronal cultures were harvested and subjected to western blotting with anti-ERα (1:500 (Santa Cruz Biotechnology), sc-542) or anti-ERβ (1:250 (Zymed, Invitrogen), Z8P). Actin was used as a loading control. *In vivo* delivery of the viral suspension (2 μl) was achieved by stereotaxic injection bilaterally into PFC with a Hamilton syringe (needle gauge 31) as we described previously.[32,35,39] Rats (19–20 days old) were used for lentivirus injection, and repeated stress exposure initiated 2 days after viral injection. Electrophysiological experiments and behavioral testing were performed at 10–12 days after the viral injection (24 h after the last stressor exposure).

Quantitative real-time RT-PCR

To compare the mRNA levels, quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used. Total RNA was isolated from rat PFC using Trizol reagent (Invitrogen) and treated with DNase I (Invitrogen) to remove genomic DNA. Then, SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) was used to obtain cDNA from the tissue mRNA, followed by the treatment with RNase H ($2\,U\,l^{-1}$) for 20 min at 37°C. Quantitative real-time RT-PCR was carried out using the iCycler iQ Real-Time PCR Detection System and iQ Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. In brief, glyceraldehyde 3-phosphate dehydrogenase was used as the housekeeping gene for quantitation of the expression of target genes (ERα, ERβ and aromatase) in samples from male vs female rats (4 weeks old). Fold changes in the target gene relative to the glyceraldehyde 3-phosphate dehydrogenase endogenous control gene was determined by: fold change $= 2^{-\Delta(\Delta CT)}$, where $\Delta CT =$ CT, target − CT, GAPDH and $\Delta(\Delta CT) = \Delta CT$, male − ΔCT, female. CT (threshold cycle) is defined as the fractional cycle number at which the fluorescence reaches 10 × the standard deviation of the baseline. A total reaction mixture of 25 μl was amplified in a 96-well thin-wall PCR plate (Bio-Rad) using the following PCR cycling parameters: 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. Quantitative real-time RT-PCR was performed in triple reactions.

Statistics

All data are expressed as the mean ± s.e.m. Experiments with two groups were analyzed statistically using unpaired Student's *t*-tests. Experiments with more than two groups were subjected to one- or two-way analysis of variance (ANOVA), followed by *post hoc* Tukey's tests.

RESULTS

Repeated stress induces differential effects on PFC-dependent behaviors and PFC glutamatergic transmission in young female vs male animals

To compare the impact of repeated stress on cognitive functions in young females and males, we subjected rats (~4 weeks old, juvenile–adolescent) to the TORM task, a cognitive process mediated by the medial PFC.[32,37] Animals were exposed to a 7-day RS (2 h per day) and examined at 24 h after stressor cessation. In males, the control groups spent much more time exploring the novel (less recent) object in the test trial (familiar recent object: 6.0 ± 0.4 s; novel object: 14.4 ± 3.2 s, $n = 6$, $P < 0.01$), whereas the stressed groups lost the preference to the novel object (familiar recent object: 15.2 ± 1.9 s; novel object: 12.1 ± 2.2 s, $n = 6$, $P > 0.05$). In females, both the control and the stressed groups spent much more time exploring the novel object in the test trial (control—familiar recent object: 6.0 ± 1.1 s; novel object: 15.0 ± 2.4 s, $n = 5$, $P < 0.01$; stressed—familiar recent object: 6.0 ± 1.0 s; novel object: 16.3 ± 2.7 s, $n = 6$, $P < 0.01$). A significant main effect was observed in the DR (an index of the object recognition memory (Figure 1a), $F_{3,22} = 19.3$, $P < 0.001$, ANOVA), and *post hoc* analysis indicated a profound impairment of recognition memory by repeated stress in male rats (DR in male control: 35.7 ± 8.0%, $n = 6$; DR in male stressed: − 13.3 ± 4.4%, $n = 6$, $P < 0.001$), but not in female rats (DR in female control: 43.2 ± 8.4%, $n = 5$; DR in female stressed: 45.5 ± 4.2%, $n = 6$, $P > 0.05$). The total exploration time in the two sample phases and the subsequent test trial was unchanged in control and stressed animals of both genders (Supplementary Figure S1A).

Next, we investigated the molecular mechanism underlying the behavioral differences of stress responses in males and females. Our recent study has shown that repeated stress impairs PFC-mediated cognitive processes by reducing PFC glutamatergic transmission,[32] so we compared AMPAR- and NMDAR-mediated synaptic currents (EPSC) in PFC pyramidal neurons from males vs females exposed to 7-day RS (2 h per day). As shown in Figures 1b and c, the amplitude of evoked AMPAR-EPSC was markedly reduced in stressed male rats ($F_{3,47} = 19.9$, $P < 0.001$, ANOVA, control: 191.9 ± 13.6 pA, $n = 12$; RS: 93.4 ± 9.9 pA, $n = 12$, $P < 0.001$), but was largely unchanged in stressed female rats (control: 223.2 ± 15.5 pA, $n = 12$; RS: 228.0 ± 15.6 pA, $n = 12$, $P > 0.05$). Similarly, a substantial impairment was found on evoked NMDAR-EPSC in stressed male rats ($F_{3,53} = 16.0$, $P < 0.001$, ANOVA, control: 221.5 ± 13.1 pA, $n = 14$; RS: 106.1 ± 9.1 pA, $n = 14$, $P < 0.001$), but not in stressed female rats (control: 185.4 ± 10.1 pA, $n = 13$; RS: 203.0 ± 17.8 pA, $n = 13$, $P > 0.05$). Moreover, the miniature EPSC (mEPSC), a synaptic response resulting from quantal release of single glutamate vesicles, was significantly reduced in PFC slices from male rats after repeated stress, but was intact in stressed females (Figures 1d and e; amplitude: $F_{3,37} = 8.2$, $P < 0.001$, ANOVA; frequency: $F_{3,37} = 7.0$, $P < 0.001$, ANOVA; male control: 16.5 ± 0.6 pA, 3.3 ± 0.3 Hz, $n = 10$; male RS: 12.9 ± 0.4 pA, 1.9 ± 0.1 Hz, $n = 10$, $P < 0.001$; female control: 15.4 ± 0.6 pA, 3.2 ± 0.2 Hz, $n = 9$; female RS: 14.8 ± 0.6 pA, 2.7 ± 0.3 Hz, $n = 9$, $P > 0.05$).

As the number of glutamate receptors at the synaptic membrane could determine the strength of glutamatergic transmission, we performed surface biotinylation and western blotting experiments to detect the surface and total level of AMPAR and NMDAR subunits in PFC slices from male vs female animals after repeated RS. The level of surface AMPAR and NMDAR subunits was substantially reduced in stressed males (Figures 1f and g; surface GluR1/2: 58–61% decrease; surface NR1/2A/2B:

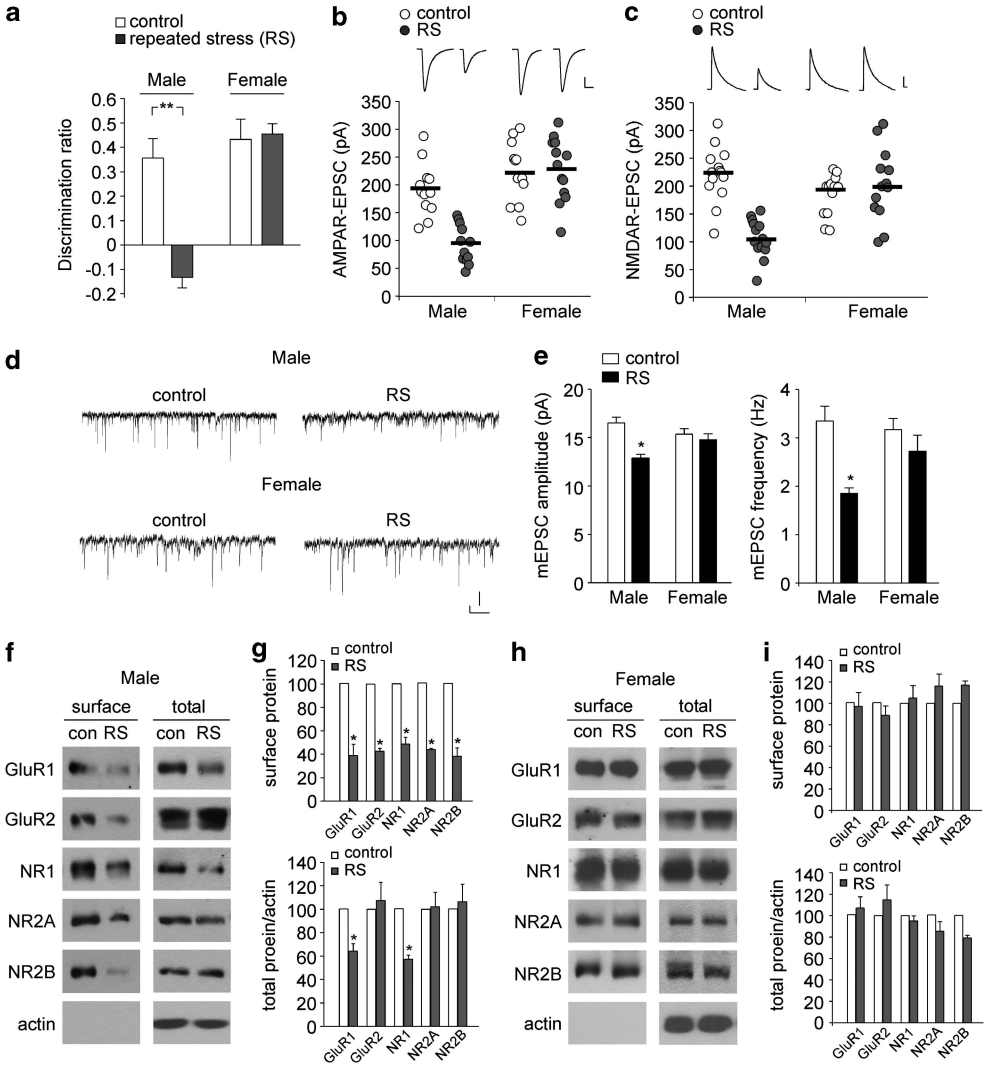

Figure 1. Repeated stress has different effects on recognition memory and PFC glutamatergic transmission in male vs female rats. (**a**) Bar graphs (mean ± s.e.m.) showing the discrimination ratio (DR) of temporal order recognition memory (TORM) tasks in control or repeatedly stressed (7-day restraint, RS) male or female rats. $^{**}P<0.005$, ANOVA. (**b**, **c**) Dot plots showing the amplitude of AMPAR-EPSC (**b**) and NMDAR-EPSC (**c**) in PFC pyramidal neurons taken from control or stressed (RS) male or female rats. Inset: representative EPSC traces. Scale bars: 50 pA, 20 ms (AMPA) or 100 ms (NMDA). (**d**, **e**) Representative mEPSC traces (**d**) and statistic summary (**e**) showing the effect of repeated stress on mEPSC amplitude and frequency in PFC neurons from male or female rats. Scale bars: 25 pA, 1 s. $^{*}P<0.01$. (**f**–**i**) Immunoblots and quantification analysis of the total and surface AMPAR and NMDAR subunits in PFC from control (con) vs stressed (RS) male (**f**, **g**) or female (**h**, **i**) rats. $^{*}P<0.01$.

55–62% decrease, $n=4$ pairs, $P<0.01$, *t*-test), but was largely unchanged in stressed females (Figures 1h and i; <10% decrease, $n=5$ pairs). The level of total GluR1 and NR1 was significantly reduced in males after repeated stress (GluR1: 36% decrease; NR1: 43% decrease, $n=5$ per group), consistent with our previous finding,[32] whereas repeated stress did not affect the total level of any glutamate receptor subunits in stressed females ($n=4$ pairs). Taken together, these data indicate that repeated stress has distinct effects on PFC glutamatergic transmission and glutamate receptor surface expression, with males being impaired and females being spared, which may explain the sexually dimorphic impact of repeated stress on recognition memory.

The contrasting responses to repeated stress that we have found were obtained from adolescent rats. To test whether the effects of repeated stress on PFC glutamatergic system also happen in adult rats, we exposed adult (p75–85) males and females to the prolonged RS (6 h per day for 8–10 days), and examined AMPAR-mediated synaptic currents. As shown in Supplementary Figure S2A, the amplitude of evoked AMPAR-EPSC was substantially decreased in stressed adult males, but was unchanged in stressed adult females ($F_{3,33}=15.4$, $P<0.001$, ANOVA; male control: 259.1 ± 13.0 pA, $n=9$; male RS: 155.1 ± 8.9 pA, $n=8$, $P<0.001$; female control: 264.8 ± 12.1 pA; $n=9$; female RS: 259.9 ± 17.8 pA, $n=8$, $P>0.05$). These changes are similar to what was observed in young males and females exposed to less severe RS (2 h per day for 5–7 days).

We also performed biochemical experiments to examine the total and surface levels of AMPAR and NMDAR subunits in adult males and females exposed to the prolonged RS. As shown in Supplementary Figure S2B, repeated stress caused a substantial reduction of the surface pool of AMPAR and NMDAR subunits in stressed adult males (40–50% decrease, $n=4$ pairs), but not in stressed adult females (<5% decrease, $n=4$ pairs). Moreover, repeated stress decreased the total level of GluR1 and NR1 in stressed adult males, whereas it did not affect the total level of all glutamate receptor subunits in stressed adult females. These data

indicate that stress could induce similar alterations of glutamate receptors in adult males and females as in young animals of both genders.

Estrogen protects against the detrimental effects of repeated stress in females

We next examined the potential mechanism underlying the contrasting effects of repeated stress on PFC functions in female vs male animals. One possibility is that estrogen influences the impact of stress. To test this, we gave female rats repeated injections of ICI182 780 (subcutaneously, 0.05 mg kg, 7 days, starting at 1 h before daily stress), an ER antagonist that penetrates the brain,[40] and examined recognition memory after repeated RS (2 h per day, 7 days). Animals were examined at 24 h after stressor cessation. As shown in Figure 2a, the ICI-injected females exposed to repeated stress lost the preference to the novel object in TORM tasks (DR in oil: $43.9 \pm 9.9\%$, $n = 7$; DR in oil + RS: $52.9 \pm 4.2\%$, $n = 5$; DR in ICI: $37.4 \pm 9.1\%$, $n = 5$; DR in ICI + RS: $-6.4 \pm 3.7\%$, $n = 5$, $F_{3,21} = 10.1$, $P < 0.001$, ANOVA). ICI injections did not alter the total exploration time in the two sample phases and the subsequent test trial (Supplementary Figure S1B).

We then examined glutamatergic responses in PFC neurons from stressed females with ICI182 780 injections. As shown in Figure 2b, in ICI-injected female rats, repeated stress caused a substantial reduction of the input/output curves of AMPAR-EPSC and NMDAR-EPSC induced by a series of stimulus intensities (AMPA: 40–60% decrease, $n = 8$–13 per group; NMDA: 50–55% decrease, $n = 9$–12 per group). Two-way ANOVA analysis revealed a significant main effect of treatment groups (AMPA: $F_{3,159} = 29.9$, $P < 0.001$; NMDA: $F_{3,155} = 29.5$, $P < 0.001$), a significant main effect of stimulation intensities (AMPA: $F_{4,159} = 96.6$, $P < 0.001$; NMDA: $F_{4,155} = 85.6$, $P < 0.001$), and a significant interaction (AMPA: $F_{12,159} = 1.8$, $P < 0.05$; NMDA: $F_{12,155} = 4.0$, $P < 0.001$).

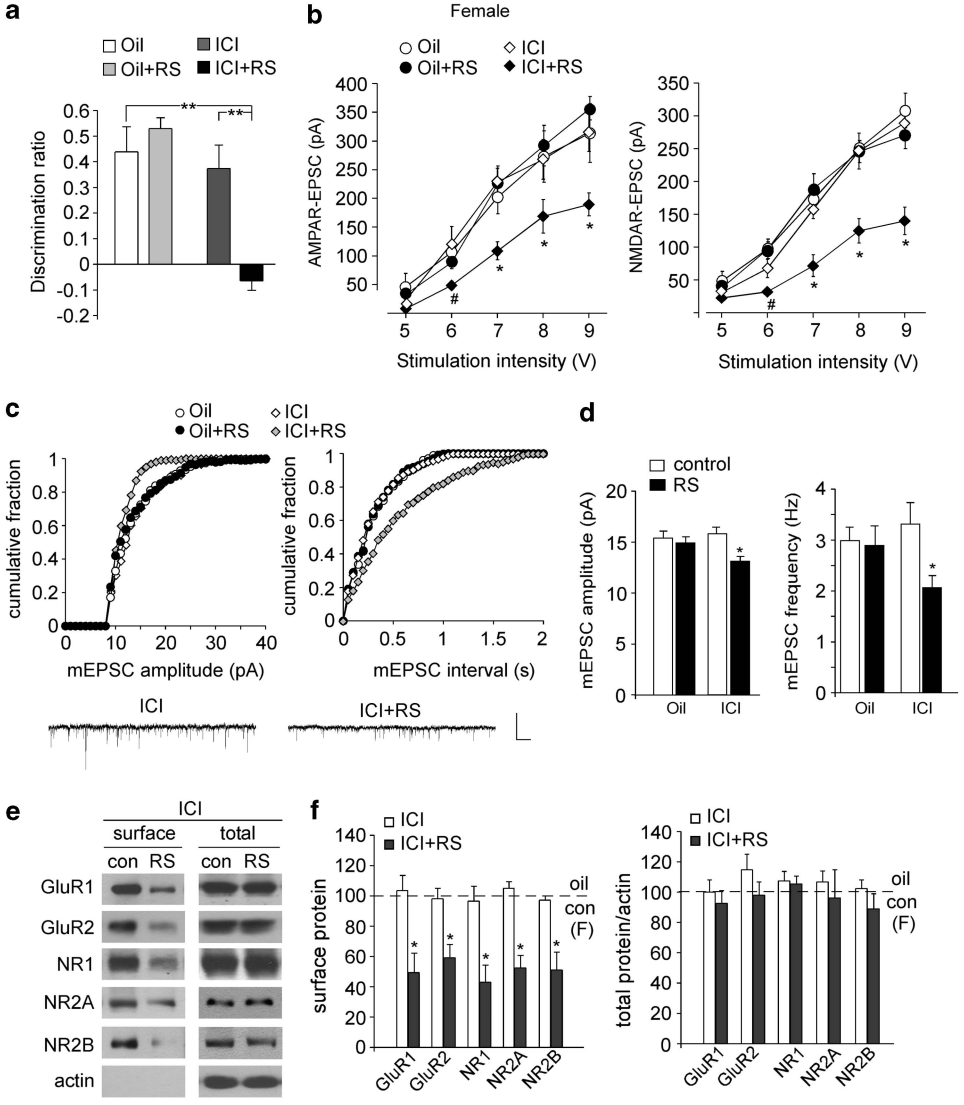

Figure 2. In females, blocking ERs unmasks the stress-induced impairment of recognition memory and PFC glutamatergic transmission. (**a**) Bar graphs (mean ± s.e.m.) showing the DR of TORM tasks in control vs repeatedly stressed females (7-day restraint, RS) with or without the injections of the ER antagonist ICI182 780 (0.05 mg kg^{-1}, subcutaneously). $^{**}P < 0.005$, ANOVA. (**b**) Summarized input–output curves of AMPAR-EPSC or NMDAR-EPSC in control vs stressed (RS) females injected with oil or ICI182 780. $^{*}P < 0.01$, $^{\#}P < 0.05$, compared to oil-injected control females. (**c**, **d**) Cumulative distribution (**c**) and bar graphs (**d**) showing the effect of repeated stress on mEPSC amplitude and frequency in females injected with or without ICI182 780. Inset: representative mEPSC traces. Scale bars: 50 pA, 1 s. $^{*}P < 0.01$. (**e**, **f**) Immunoblots (**e**) and quantification analysis (**f**) of the total and surface AMPAR and NMDAR subunits in PFC from control (con) vs stressed (RS) females injected with ICI182 780. $^{*}P < 0.01$, compared to oil-injected control females (indicated with dotted lines in **f**).

Post hoc multiple comparison tests revealed that the ICI-injected stressed group had significantly lower responses than other groups at all points ($P<0.05$ or $P<0.01$), except for the weakest stimuli. Moreover, mEPSC was significantly reduced in ICI-injected female rats after repeated stress, but not in oil-injected females (Figures 2c and d; amplitude: $F_{3,32}=5.6$, $P<0.01$, ANOVA; frequency: $F_{3,32}=3.5$, $P<0.05$, ANOVA; ICI: 15.8 ± 0.6 pA; 3.3 ± 0.4 Hz, $n=10$; ICI + RS: 13.1 ± 0.4 pA, 2.1 ± 0.2 Hz, $n=10$; $P<0.01$; oil: 15.4 ± 0.6 pA, 3.0 ± 0.3 Hz, $n=7$; oil + RS: 15.0 ± 0.6 pA, 2.9 ± 0.4 Hz, $n=6$, $P>0.05$).

We also detected the surface and total level of AMPAR and NMDAR subunits in PFC slices from ICI182 780-injected females after repeated RS. As shown in Figures 2e and f, a significant reduction was found in stressed females with ICI182 780 injections (surface GluR1/2: 41–51% decrease; surface NR1/2A/2B: 48–57% decrease, $n=5$ pairs, $P<0.01$, *t*-test). Repeated stress did not affect the total level of glutamate receptor subunits in ICI-injected females ($n=4$ pairs). Taken together, it suggests that estrogen protects females against the detrimental effects of repeated stress on PFC glutamatergic transmission and PFC-dependent cognitive process.

Estrogen prevents the stress-induced impairments in males

To further confirm the protective role of estrogen, we gave male rats repeated injections of the ER agonist estradiol (0.1 mg kg, subcutaneously, starting at 1 h before daily stress), and examined recognition memory after repeated RS (examined at 24 h after stressor cessation). As shown in Figure 3a, the stress-induced impairment of TORM was prevented in estradiol-injected males (DR in oil: $43.7\pm11.3\%$, $n=7$; DR in oil + RS: $-20.5\pm3.5\%$, $n=7$; DR in estradiol: $39.8\pm7.0\%$, $n=6$; DR in estradiol + RS: $48.0\pm12.2\%$, $n=6$, $F_{3,25}=13.2$, $P<0.001$, ANOVA). Estradiol injections did not alter the total exploration time in the two sample phases and the subsequent test trial (Supplementary Figure S1C).

Electrophysiological recordings also showed that the stress-induced impairment of AMPAR-EPSC or NMDAR-EPSC was largely blocked in estradiol-injected male rats (Figure 3b; <15% decrease, $n=7$–12 per group), but not in oil-injected males (~50% decrease, $n=13$–19 per group). Two-way ANOVA analysis revealed a significant main effect of treatment groups (AMPA: $F_{3,216}=70.0$, $P<0.001$; NMDA: $F_{3,197}=87.4$, $P<0.001$), a significant main effect of stimulation intensities (AMPA: $F_{4,216}=170.1$, $P<0.001$; NMDA: $F_{4,197}=95.5$, $P<0.001$) and a significant interaction (AMPA: $F_{12,216}=6.0$, $P<0.001$; NMDA: $F_{12,197}=4.4$, $P<0.001$). *Post hoc* multiple comparison tests revealed that the oil-injected stressed group had significantly lower responses than other groups at all points ($P<0.01$), except for the weakest stimuli. Estradiol itself caused a modest but significant enhancement of eEPSC at strong stimulus points (AMPA: 25–40% increase; NMDA: 23–45% increase, $n=8$–10 per group, $P<0.05$ compared with oil control). Moreover, mEPSC was intact in stressed males injected with estradiol, but not those injected with oil (Figures 3c and d; amplitude: $F_{3,27}=29.5$, $P<0.001$, ANOVA; frequency: $F_{3,27}=15.3$, $P<0.001$, ANOVA; estradiol: 16.8 ± 0.6 pA, 6.0 ± 0.7 Hz, $n=7$; estradoil + RS: 16.2 ± 0.8 pA, 5.8 ± 0.3 Hz, $n=5$, $P>0.05$; oil: 13.9 ± 0.5 pA, 3.6 ± 0.5 Hz, $n=9$; oil + RS: 9.9 ± 0.4 pA, 1.6 ± 0.2 Hz, $n=7$, $P<0.01$). Estradiol itself also significantly increased mEPSC (Figure 3d; ~21% increase in amplitude, ~67% increase in frequency, $P<0.05$, ANOVA, $n=7$–9 per group).

Biochemical experiments showed that the stress-induced reduction of surface AMPAR and NMDAR subunits was largely blocked in estradiol-injected male rats (Figures 3e and f; <5% decrease, $n=4$ per group). The significant reduction of total GluR1 and NR1 in stressed males was also blocked by estradiol injections (Figures 3e and f; <5% decrease, $n=5$ per group). Estradiol itself caused a 20–40% increase of the surface and total level of AMPAR and NMDAR subunits in PFC slices ($P<0.05$, ANOVA compared with oil control; Figures 3e and f). Taken together, it suggests that exogenous estrogen prevents the detrimental effects of repeated stress on PFC glutamatergic transmission and PFC-dependent cognitive process in males.

ERα mediates the protective effect of estrogen against repeated stress

Given the role of estrogen in protecting females against the detrimental effects of repeated stress, we would like to know which ER is potentially involved. Thus, we performed RNA interference-mediated knockdown of ERα or ERβ, and examined the impact of repeated stress on glutamatergic transmission in PFC neurons from female rats. ERα or ERβ shRNA was expressed in a lentiviral vector that also independently expressed GFP. As illustrated in Figure 4a, ERα or ERβ shRNA lentivirus caused a specific and effective suppression of the expression of these ERs in cortical cultures (~50% decrease, $n=6$, $P<0.01$, ANOVA). ERα or ERβ shRNA lentivirus was delivered *in vivo* to female rat frontal cortex via a stereotaxic injection.[32,39] The GFP-labeled neurons, which should also express ERα or ERβ shRNA, showed normal morphological structures (Figure 4b). As shown in Figures 4c–e, repeated stress caused a significant downregulation of the AMPAR-EPSC amplitude in ERα shRNA lentivirus-injected, but not GFP-injected, female rats (32–46% decrease, $n=7$–12 per group, $F_{3,175}=36.0$ for treatment groups, $P<0.001$, two-way ANOVA), whereas repeated stress had little effect in ERβ shRNA lentivirus-injected female rats (<10% decrease, $n=8$–11 per group, $F_{3,97}=1.3$ for treatment groups, $P>0.05$, two-way ANOVA). It suggests that ERα mediates the protective effect of estrogen against stress-induced downregulation of AMPAR responses in PFC neurons from female rats.

To further confirm the protective role of ERα, we examined recognition memory in stressed females with ERα knockdown in PFC. As shown in Figure 4f, after exposure to repeated RS, females with PFC injection of ERα shRNA lentivirus lost the preference to the novel object in TORM tasks, in contrast to the intact recognition memory in GFP lentivirus-injected females (DR in GFP: $46.6\pm5.6\%$, $n=5$; DR in GFP + RS: $45.4\pm4.0\%$, $n=5$; DR in ERα shRNA: $45.6\pm10.1\%$, $n=5$, DR in ERα shRNA + RS: $4.2\pm4.6\%$, $n=5$, $F_{3,19}=10.3$, $P<0.001$, ANOVA). The total exploration time in the two sample phases and the subsequent test trial was not altered by ERα shRNA lentivirus delivery to the PFC (Supplementary Figure S1D).

We further examined the effects of repeated stress in ovariectomized females (4 weeks old). Surprisingly, in ovariectomized females, repeated RS had little effect on AMPAR-EPSC (<10% decrease, $n=7$–10 per group, $F_{1,67}=0.2$ for treatment groups, $P>0.05$, two-way ANOVA; Supplementary Figure S3A), and failed to alter the surface or total level of GluR1 and NR1 subunits ($n=3$ pairs; Supplementary Figure S3B), similar to what was observed in gonadally intact young females after stress (Figure 1). It suggests that it may be brain-produced estrogen that influences the synaptic effects of repeated stress.

The estrogen synthesis enzyme aromatase is crucial for the sexually dimorphic effects of repeated stress.

To determine the role of endogenous circulating estrogen, as well as the potential involvement of estrogen produced in neural tissues, we used the aromatase inhibitor formestane, which blocks synthesis of estrogen both peripherally and centrally. Formestane was injected into females (subcutaneously 2 mg kg, 7 days, starting at 1 h before daily stress), and PFC neurons were examined after repeated RS (2 h per day, 7 days). As shown in Figure 5a, repeated stress caused a substantial reduction of the input/output curves of AMPAR-EPSC in formestane-injected female rats (40–50% decrease, $n=11$–13 per group, $F_{1,95}=39.5$ for treatment groups, $P<0.001$, two-way ANOVA). A significant

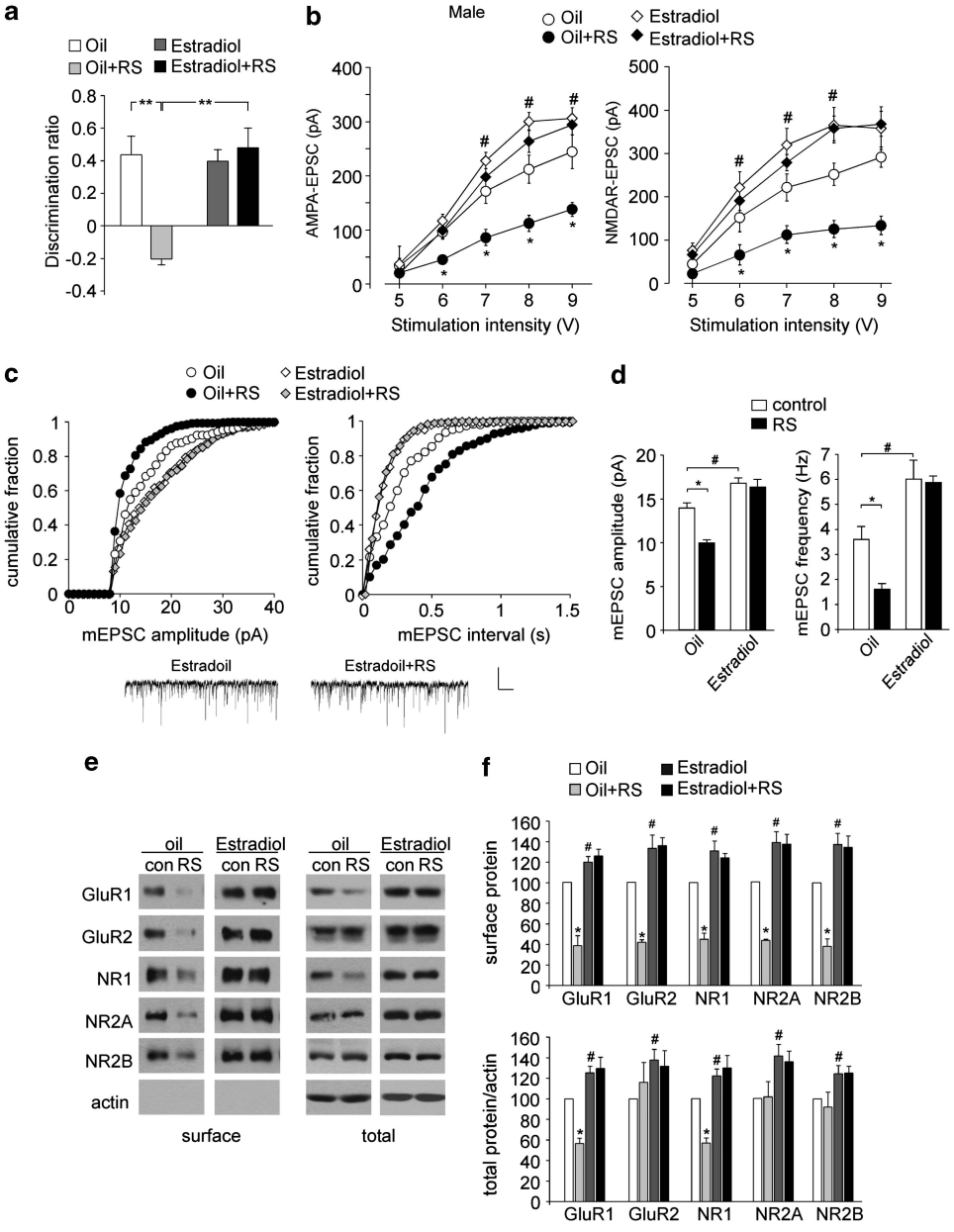

Figure 3. In males, administration of an ER agonist prevents the stress-induced impairment of recognition memory and PFC glutamatergic transmission. (**a**) Bar graphs (mean ± s.e.m.) showing the DR of TORM tasks in control vs repeatedly stressed males (7-day restraint, RS) with or without the injections of the ER agonist estradiol (0.1 mg kg^{-1}, subcutaneously). $^{**}P<0.005$, ANOVA. (**b**) Summarized input–output curves of AMPAR-EPSC or NMDAR-EPSC in control vs stressed (RS) males injected with estradiol or oil control. $^{*}P<0.01$, $^{\#}P<0.05$, compared to oil-injected control males. (**c**, **d**) Cumulative distribution (**c**) and bar graphs (**d**) showing the effect of repeated stress on mEPSC amplitude and frequency in males injected with or without estradiol. Inset: representative mEPSC traces. Scale bars: 25 pA, 1 s. $^{*}P<0.001$. (**e**, **f**) Immunoblots (**e**) and quantification analysis (**f**) of the total and surface AMPAR and NMDAR subunits in PFC from control (con) vs stressed (RS) males injected with oil or estradiol. $^{*}P<0.01$, $^{\#}P<0.05$, compared to oil-injected control males.

reduction was also found with the surface expression of glutamate receptors in stressed females with formestane injections (Figures 5b and c; surface GluR1/2: 63–68% decrease; surface NR1/2A/2B: 39–55% decrease, $n=4$–7 pairs, $P<0.01$ or $P<0.05$, *t*-test).

Recognition memory was also examined in formestane-injected females after repeated RS. As shown in Figure 5d, stressed females with formestane injections lost the preference to the novel object in TORM tasks (DR in oil: 42.0 ± 12.8%, $n=6$; DR in oil + RS: 53.0 ± 3.4%, $n=6$; DR in formestane: 47.9 ± 13.5%, $n=6$; DR in formestane + RS: − 22.6 ± 9.1%, $n=6$, $F_{3,23}=11.4$, $P<0.001$, ANOVA). The total exploration time in the two sample phases and the subsequent test trial was not altered by formestane injections (Supplementary Figure S1E).

Finally, we compared the expression level of ERα, ERβ and aromatase in PFC of males and females (4 weeks old). As shown in Figure 5e, the mRNA levels of ERα and ERβ were similar in both genders (ERα—male: 1.3 ± 0.2, female: 1.2 ± 0.1, $n=6$; ERβ—male: 1.1 ± 0.1, female: 1.1 ± 0.1, $n=6$), but the mRNA level of aromatase was significantly higher in females than in males (male: 1.0 ± 0.02; female: 1.6 ± 0.2, $n=10$, $P<0.05$, *t*-test). Taken together, these results suggest that female PFC has access to estrogen from an

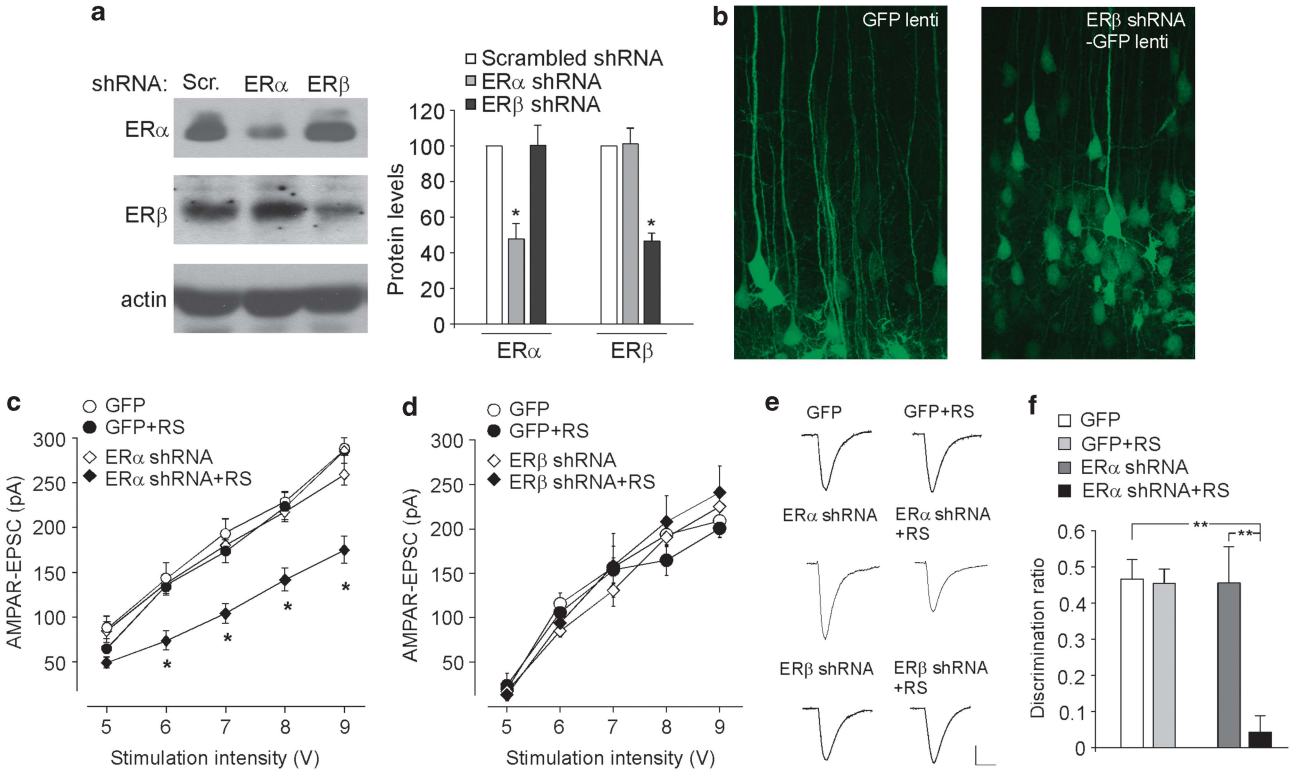

Figure 4. Knockdown of ERα *in vivo* reveals the stress-induced impairment of PFC glutamatergic transmission in female rats. (**a**) Representative western blots and quantification analysis in cortical cultures infected with an ERα or ERβ shRNA lentivirus. $^{*}P<0.01$. (**b**) Confocal images of cortical slices from female rats with stereotaxical injection of ERβ shRNA lentivirus or GFP control lentivirus. (**c**, **d**) Summarized input–output curves of AMPAR-EPSC in control vs stressed (7-day restraint, RS) females infected with GFP, ERα (**c**) or ERβ (**d**) shRNA lentivirus. $^{*}P<0.01$, compared to GFP-infected control females. (**e**) Representative EPSC traces. Scale bars: 50 pA, 20 ms. (**f**) Bar graphs (mean ± s.e.m.) showing the DR of TORM tasks in control vs repeatedly stressed females (7-day restraint, RS) with the PFC injection of GFP or ERα shRNA lentivirus. $^{**}P<0.005$, ANOVA.

unknown source, possibly the brain itself, which acts on ERα to protect females against the detrimental effects of repeated stress on glutamatergic signaling and PFC functions.

DISCUSSION

A large number of studies indicate that gonadal hormone differences at the early developmental stage of both genders exert permanent influences on brain structure and function.[41,42] Converging evidence suggests that females and males exhibit different biochemical, cellular and behavioral effects of stress.[4,43,44] Here we show that the sex differences in behavioral responses to repeated stress are causally linked to the differential effects of corticosteroid stress hormones on PFC excitatory transmission and glutamate receptor surface expression, which are determined by endogenously generated estrogen. The detrimental effects of repeated stress are revealed in females when estrogen signaling is blocked, whereas they are blocked in males when estrogen signaling is activated.

Our findings are in agreement with previous reports on the stress resistance of females in many domains.[16] In contrast to the impaired cognition in male rodents after chronic stress, female rodents show unaffected or enhanced performance on the same memory tasks after the same stress.[7,12,45,46] Other work suggests that females respond differently to stress when estrogen is present.[47,48] In humans, some stress-related mental disorders, such as major depression, are more prevalent in women,[49] but men are also stress-sensitive and show a greater prevalence of substance abuse, antisocial behavior and schizophrenia. It is consistent with evidence for the subtle but widespread brain sex differences and the notion that men and women differ in coping strategies to challenges.[50] Although stress may precipitate a variety of emotional and cognitive difficulties in the susceptible individuals,[51] for the average individuals, stress does not necessarily lead to mental disorders. What determines resilience or susceptibility is still unknown, and remains a very active area of research.[52] Genetic factors may modulate an individual's response to stress. For instance, recent findings have demonstrated that carrying the val66met allele of the *brain-derived neurotrophic factor* gene alters the vulnerability to stress and responses to antidepressants.[53] Epigenetic mechanisms, which control chromatin remodeling and gene expression, could also influence stress sensitivity.[54] In addition, severe or chronic stress across the childhood–adolescent period could trigger anxiety- and depression-like behaviors.[55,56] The repeated stress paradigms used in our studies[32] are more similar to what could be considered 'modest subchronic' stress, which is unlikely to cause depression or anxiety in the majority of cases.

Sex hormones have been found to modulate adaptive structural and functional plasticity.[44] For example, ovarian steroid estradiol can increase the densities of dendritic spines and synapses on hippocampal CA1 pyramidal cells in female rats via a mechanism involving the increased sensitivity to NMDAR-mediated synaptic input.[57,58] Ovariectomy decreases synaptic markers within the hippocampus and PFC.[59,60] In addition, estrogen also modulates functional measures of synaptic connectivity in the brain, with a

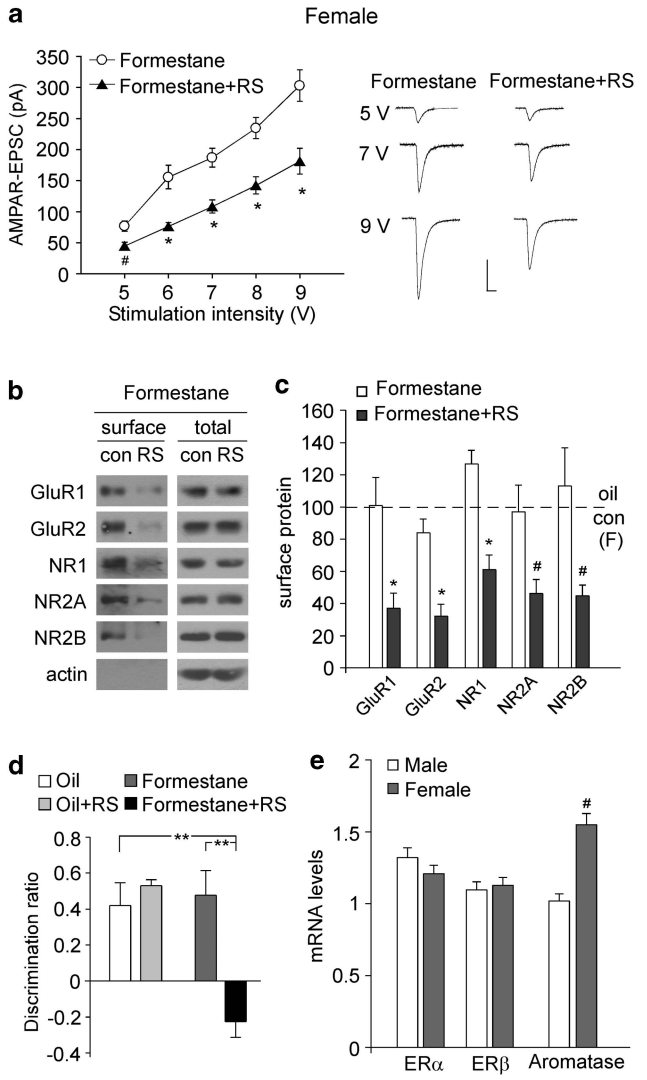

Figure 5. The estrogen synthesis enzyme aromatase is responsible for the sexually dimorphic effects of repeated stress. (**a**) Summarized input–output curves of AMPAR-EPSC in control vs repeatedly stressed (7-day restraint, RS) females injected with oil or the aromatase inhibitor formestane (2 mg kg^{-1}, subcutaneously). Inset: representative EPSC traces. Scale bars: 100 pA, 20 ms. $^{*}P<0.01$, $^{\#}P<0.05$. (**b**, **c**) Immunoblots (**b**) and quantification analysis (**c**) of the total and surface AMPAR and NMDAR subunits in PFC from control (con) vs stressed (RS) females injected with formestane. The bar graphs (**c**) were plotted against the control females injected with oil. $^{*}P<0.01$, $^{\#}P<0.05$. (**d**) Bar graphs (mean ± s.e.m.) showing the DR of TORM tasks in control vs repeatedly stressed females (7-day restraint, RS) with or without the injections of formestane (2 mg kg^{-1}, subcutaneously). $^{**}P<0.005$, ANOVA. (**e**) Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) data on the mRNA level of ERα, ERβ and aromatase in PFC from male vs female rats (4 weeks old). $^{\#}P<0.05$.

reduction of long-term potentiation induction in the hippocampus of ovariectomized animals, and a facilitation of long-term potentiation by estradiol treatment.[61,62] Activation of ERβ increases key synaptic proteins, enhances hippocampal long-term potentiation, increases dendritic branching and spine density, and improves hippocampus-dependent cognition.[63] Consistent with these positive effects of estrogen on synapses, we show here that estradiol injections lead to increased AMPAR and NMDAR subunit expression and synaptic responses in PFC neurons from male animals.

There are clear differences between males and females in stress-induced changes in the brain, and in some cases they link to female gonadal hormones.[64] In response to chronic stress or corticosterone administration, contrary to males, females are resistant to the shrinkage of CA3 apical dendrites.[45,65] Estradiol supplementation given before stress in ovariectomized females increases the number of hippocampal spine synapses.[66] In layer II–III medial PFC pyramidal neurons, repeated RS decreases apical dendritic branch number and length in males, but increased these measures in intact and estrogen-treated females but not in ovariectomized females.[67] This finding was further refined to show that, for medial PFC neurons projecting to basolateral amygdala, stress increases the length and branching of dendrites in females in an estrogen-dependent manner, but not males; there were also estrogen-dependent effects on spine density in female medial PFC neurons that were independent of stress.[68] Corroborating with our discovery of the role of estrogen in females' stress resilience, estrogen also shows antianxiety and antidepressant-like effects in animal models, which are dependent on the utilized regimen of estrogen and interactions with the hypothalamic-pituitary-adrenal axis.[69]

This study indicates that corticosterone and estrogen could interact at the level of glutamate receptors and excitatory synaptic transmission to influence functional plasticity in PFC neurons, supporting the notion that glutamatergic system is the key molecular player in normal cognitive processes and mental disorders.[29,30,70–72] In males exposed to repeated stress, GR activation reduces the total and surface levels of glutamate receptors by increasing the ubiquitin/proteasome-mediated degradation of AMPAR and NMDAR subunits.[32] In females exposed to repeated stress, the surface and total levels of AMPAR and NMDAR subunits do not show the marked reduction as in stressed males. Blocking ERs in stressed females unmasks the reducing effect on the surface levels of glutamate receptors, whereas administration of estradiol in stressed males leads to the increased levels of both total and surface glutamate receptors. Thus, corticosterone and estrogen may mainly affect glutamate receptor membrane insertion in females, and affect glutamate receptor expression/turnover in males.

A surprising finding in this study is the lack of differences in the effects of repeated stress in ovariectomized vs gonadally intact prepubertal females, which suggests that it may be estradiol produced in the female brain[21,73] that interferes with the synaptic action of corticosteroids under conditions of subchronic stress. Consistently, it has been shown that estrogen can be synthesized by aromatase localized in neurons from endogenous cholesterol.[74] There exists the possibility that locally synthesized estrogen in the brain can also impact the structure and function of neural circuits.[75–79] Ischemic neuroprotection in females has been attributed to the local, nongonadal estrogen, which may be aromatized from precursor androgens.[80] In this study, we demonstrate that treatment of females with the aromatase inhibitor formestane, which readily crosses the blood–brain barrier and can inhibit central estrogen production, results in a more male-like neural and behavioral phenotype. In addition, knockdown of ERα in PFC of females led to the loss of protection against the physiological and biochemical effects of repeated stress, further suggesting the role of brain estrogen in this process. The differential expression of estrogen synthesis enzyme aromatase in PFC neurons of young male vs females is another factor to be considered in the efficacy of estrogen in females. Our results suggest that the female PFC has an endogenous capacity to generate estrogen that provides protection against subchronic repeated stress.

In conclusion, these results suggest that estrogen protects against the detrimental effects of repeated stress, mediated

in part by glucocorticoids, on glutamatergic transmission and PFC-dependent cognition, and that this may underlie the stress resilience of females.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)