ANESTHESIOLOGY

Distinct Function of Estrogen Receptors in the Rodent Anterior Cingulate Cortex in Pain-related Aversion

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Estrogen produced within the central nervous system may modulate pain in both males and females
- Estrogen receptors within the rostral anterior cingulate cortex modulate pain-related behaviors in rodent pain models

What This Article Tells Us That Is New

- Blockade of the estrogen receptor-β but not the estrogen receptor-α reduced pain-related aversion in rats, a model of the affective components of pain
- Administration of an estrogen receptor-β agonist to the rostral anterior cingulate cortex caused conditioned place aversion without altering mechanical or thermal sensitivity
- Estrogen receptor-β may be a key receptor controlling the affective components of pain-related behaviors in laboratory models

ABSTRACT

Background: Brain-derived estrogen is implicated in pain-related aversion; however, which estrogen receptors mediate this effect remains unclear. This study hypothesized that the different estrogen receptors in the rostral anterior cingulate cortex play distinct roles in pain-related aversion.

Methods: Formalin-induced conditioned place avoidance and place escape/avoidance paradigms were used to evaluate pain-related aversion in rodents. Immunohistochemistry and Western blotting were used to detect estrogen receptor expression. Patch-clamp recordings were used to examine *N*-methyl-D-aspartate–mediated excitatory postsynaptic currents in rostral anterior cingulate cortex slices.

Results: The administration of the estrogen receptor-β antagonist 4-(2-phenyl-5,7-bis [trifluoromethyl] pyrazolo [1,5-a] pyrimidin-3-yl) phenol (PHTPP) or the G protein–coupled estrogen receptor-1 antagonist (3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta [c] quinolone (G15) but not the estrogen receptor-α antagonist 1,3-bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole dihydrochloride (MPP) into the rostral anterior cingulate cortex blocked pain-related aversion in rats (avoidance score, mean ± SD: 1,3-bis [4-hydroxyphenyl]-4-methyl-5-(4-[2-piperidinylethoxy] phenol)-1H-pyrazole dihydrochloride (MPP): 47.0 ± 18.9%, 4-(2-phenyl-5,7-bis [trifluoromethyl] pyrazolo [1,5-a] pyrimidin-3-yl) phenol (PHTPP): −7.4 ± 20.6%, and [3aS*,4R*,9bR*]-4-[6-bromo-1,3-benzodioxol-5-yl]-3a,4,5,9b-3H-cyclopenta [c] quinolone (G15): −4.6 ± 17.0% *vs.* vehicle: 46.5 ± 12.2%; n = 7 to 9; $P < 0.0001$). Consistently, estrogen receptor-β knockdown but not estrogen receptor-α knockdown by short-hairpin RNA also inhibited pain-related aversion in mice (avoidance score, mean ± SD: estrogen receptor-α–short-hairpin RNA: 26.0 ± 7.1% and estrogen receptor-β–short-hairpin RNA: 6.3 ± 13.4% *vs.* control short-hairpin RNA: 29.1 ± 9.1%; n = 7 to 10; $P < 0.0001$). Furthermore, the direct administration of the estrogen receptor-β agonist 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN) or the G protein–coupled estrogen receptor-1 agonist (±)-1-([3aR*,4S*,9bS*]-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl)-ethanone (G1) into the rostral anterior cingulate cortex resulted in conditioned place avoidance (avoidance score, mean ± SD: 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN): 35.3 ± 9.5% and (±)-1-([3aR*,4S*,9bS*]-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta [c]quinolin-8-yl)-ethanone (G1): 43.5 ± 22.8% *vs.* vehicle: 0.3 ± 14.9%; n = 8; $P < 0.0001$) but did not affect mechanical or thermal sensitivity. The activation of the estrogen receptor-β/protein kinase A or G protein–coupled estrogen receptor-1/protein kinase B pathway elicited the long-term potentiation of *N*-methyl-D-aspartate–mediated excitatory postsynaptic currents.

Conclusions: These findings indicate that estrogen receptor-β and G protein–coupled estrogen receptor-1 but not estrogen receptor-α in the rostral anterior cingulate cortex contribute to pain-related aversion by modulating *N*-methyl-D-aspartate receptor–mediated excitatory synaptic transmission.

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Accumulating evidence has shown that brain-derived estrogen plays critical roles in the central nervous system.[1] Neurons in the central nervous system are targets of estrogen, which regulates neural development, plasticity, and neuroprotective actions in behavioral and cognitive functions.[2,3] Our previous study found that brain-derived estrogen in the rostral anterior cingulate cortex is required for the acquisition of pain-related aversion. Noxious stimulation

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with formalin elicits an analogous increase in the extracellular estrogen concentration in both sexes. The inhibition of local estrogen synthesis by the aromatase inhibitor androstatrienedione or the nonselective blockade of estrogen receptors prevents the induction of formalin-induced conditional place avoidance without sex specificity. Exogenous estrogen rapidly enhances excitatory synaptic transmission in pyramidal neurons in the rostral anterior cingulate cortex. These results indicate that nociceptor-driven estrogen in the rostral anterior cingulate cortex elicits pain-related aversion by modulating *N*-methyl-D-aspartate (NMDA) receptor function.[4] Growing evidence suggests that selectively targeting specific areas of the brain, estrogen receptor subtypes, and signaling pathways may be important for optimizing treatment for estrogen-related diseases.[5] Thus, elucidating the complex actions of estrogen on different estrogen receptors and downstream signaling pathways in the rostral anterior cingulate cortex is important for further understanding the mechanism of estrogen-mediated pain-related aversion.

Estrogen receptors are known to contain intracellular nuclear estrogen receptors and membrane estrogen receptors. The classical estrogen receptors estrogen receptor-α and estrogen receptor-β are located in both the nucleus and membrane, where they have distinct effects. Nuclear estrogen receptors generally mediate slow transcription-related genomic action, whereas membrane estrogen receptors normally mediate rapid nongenomic action.[6–9] In addition to estrogen receptor-α and estrogen receptor-β, G protein–coupled estrogen receptor-1 (formerly known as GPR30) is also a membrane estrogen receptor, and it only regulates the rapid nongenomic responses in cells.[9–12] Our previous study showed that the nonselective estrogen receptor antagonist ICI 182,780 completely blocks formalin-induced conditional place avoidance, whereas 17β-estradiol–bovine serum albumin (a membrane-impermeable estrogen) can mimic 17β-estradiol–induced aversion in the anterior cingulate cortex, suggesting that estrogen receptors may be involved in pain-related aversion.[4] Studies have demonstrated that estrogen receptor-α, estrogen receptor-β, and G protein–coupled estrogen receptor-1 mediate different brain functions.[13,14] For instance, estrogen receptor-α and G protein–coupled estrogen receptor-1 are implicated in neuroprotection against ischemic injury, whereas estrogen receptor-β is required for excitatory synaptic transmission, spatial memory, anxiety, and depressive behaviors.[15–18] We therefore hypothesized that the different subtypes of estrogen receptors in the rostral anterior cingulate cortex may also play distinct roles in pain-related aversion *via* diverse downstream mechanisms.

In the present study, using formalin-induced conditional place avoidance and place escape/avoidance paradigms, which are generally used to evaluate inflammatory pain- and neuropathic pain-related aversive emotion, respectively, in rodents, we investigated whether and how the different subtypes of estrogen receptors and their downstream signaling pathways in the rostral anterior cingulate cortex regulate pain-related aversion. Solving these questions may provide us with new strategies for developing therapeutics for estrogen-related disorders, especially pain-related emotional disorders.

Materials and Methods

Animals and Reagents

Sprague–Dawley rats (adult: 2 to 3 months old, 200 to 220 g; young: 2 to 3 weeks old, 50 to 80 g, for electrophysiologic recording only) and C57BL/6 mice (8 to 12 weeks old, 20 to 25 g) were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Science, Shanghai, China. CaMK2α::EYFP mice (8 to 12 weeks old, 20 to 25 g) were bred by crossing CaMK2α-Cre mice (catalog No. 005359 from Jackson Laboratory, USA) and R26R-EYFP mice (catalog No. 006148 from Jackson Laboratory). A total of 300 male and female rats and 46 male mice were used for *in vivo* and *in vitro* studies (table 1). All animals were housed under a 12:12-h light-dark cycle in an air-conditioned room (23 ± 2°C) with water and food *ad libitum*. The animals were numbered, randomly assigned to different experimental groups, and then tested in sequential order. All animal experiments were approved by the Committee on the Use of Animal Experiments of Fudan University (permit No. SYXK 2009-0082) and followed the policies on the use of laboratory animals issued by the International Association for the Study of Pain (Washington, D.C.). After the experiments, the animals were euthanized *via* carbon dioxide inhalation. All the behavioral tests, electrophysiologic recordings, and Western blot and immunohistochemical experiments described herein were performed by experimenters who were blinded to the treatments. The primary outcome was the effect of pharmacologic and genetic interventions involving different estrogen receptors in the rostral anterior cingulate cortex on pain-related aversion. No analgesics were used in this study.

1,3-Bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole dihydrochloride (MPP) and PHTPP were purchased from Tocris Bioscience (United Kingdom). (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta [c] quinolone (G15), 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN), (±)-1-[(3aR*,4S*,9bS*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta [c]quinolin-8-yl]-ethanone, 17β-estradiol (a main form of estrogen), Rp–adenosine-3′,5′-cyclic monophosphorothiote triethyammonium salt hydrate (cAMP), (1S,6bR,9aS, 11R,11bR) 11-(acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3H-furo(4,3,2-de)indeno(4,5,-h)-2-)]-2-benzopyran-3,6,9-trione (wortmannin), sesame oil, and dimethyl sulfoxide were purchased from Sigma (USA). 17β-Estradiol was dissolved in sesame oil. MPP (1 mM), PHTPP (1 mM), G15

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Table 1. Numbers of Animals Used in the Different Experiments

Experiment	Sample Size	Groups (n)	Samples (n)	Rats/Mice (n)	Weight (Rat/Mouse)
Behavioral test	n = 7–10	34	236 rats/42 mice	236/42	200-220 g/20–25 g
Immunohistochemistry	n = 4	2	4 rats/4 mice	4/4	200-220 g/20–25 g
Western blot	n = 4–8	7	22 rats	22/0	200–220 g
Patch-clamp recording	n = 6–11 neurons	7	63 neurons	26/0	50–80 g
Real-time polymerase chain reaction	n = 4–8	2	12 rats	4 male, 8 female/0	200–220 g
Total number of animals				300/46	

A total of 300 Sprague–Dawley rats (8 females and 292 males) and 46 male mice (42 wild-type C57BL/6 mice and 4 CaMK2α::EYFP mice were used in this study.

(1 mM), PPT (1 mM), DPN (5 mM), G1 (5 mM), wortmannin (1 mM), and Rp-cAMP (1 mM) were dissolved in dimethyl sulfoxide. Stock solutions of the drugs were diluted in normal saline or artificial cerebrospinal fluid.

Short-hairpin RNA and Virus Preparation

Estrogen receptor-α and estrogen receptor-β short-hairpin RNA sequences were designed based on the works of Ogawa and coworkers.[19,20] Estrogen receptor-α–short-hairpin RNA (shERα, Esr1, 5′-GGCATGGAGCATCTCTACA-3′), estrogen receptor-β–short-hairpin RNA (shERβ, Esr2, 5′-GCCACGAATCAGTGTACCAT-3′), or short-hairpin control (the scrambled sequence) was ligated into the designated plasmid vector construct (pAOV–CaMK2α–MCS–EGFP–3FLAG), which was designed to coexpress enhanced green fluorescent protein and the ligated short-hairpin RNA driven by the CaMK2α promoter. Then the reconstructed vector was packaged in adeno-associated virus 9 (AAV9). The reconstruction and packaging processes were completed by the Obio Technology Corp., Ltd. (China).

Surgery and Drug Infusion

Spared nerve injury was performed according to previous protocols.[21] After anesthesia with pentobarbital (intraperitoneal 45 mg/kg), the common peroneal and tibial nerves, two of the three terminal branches of the sciatic nerve, were ligated with a 4-0 silk suture and cut, and 3-mm portions of the nerves were removed. The third branch of the sciatic nerve, the sural nerve, was left intact. Sham-operated rats underwent nerve exposure but not ligation or removal.

Cannula implantation and microinjection were performed in the rats as previously described.[4] At the end of the experiments, the brains were sectioned for neutral red staining to verify the cannula position and injection site.

For virus injections, the mice were anesthetized with sodium pentobarbital (intraperitoneal 50 mg/kg) and securely placed into a mouse stereotaxic device so that bregma and lambda were horizontal. AAV9–CaMK2α–shERα–EGFP (2.10×10^{13} vector genomes/ml), AAV9–CaMK2α– shERβ–EGFP (3.37×10^{12} vector genomes/ml), or AAV9–CaMK2α–short-hairpin control–EGFP (1.53×10^{13} vector genomes/ml) was bilaterally injected (0.3 μl/hemisphere, 0.05 μl/min) into the rostral anterior cingulate cortex (from bregma: anteroposterior +1.6 mm, mediolateral ± 0.3 mm, dorsoventral −1.7 mm) according to the Paxinos and Watson mouse brain atlas with a Nanoliter 2010 injector (WPI, USA). Experiments were performed 2 to 3 weeks after virus infection. At the end of the experiments, the brains were sectioned to verify the injection site and the knockdown efficiency of the short-hairpin RNAs.

Conditioned Place Avoidance

Formalin-induced conditioned place avoidance was evaluated as described previously with slight modifications.[4,22] The apparatus consisted of three compartments: two large conditioning compartments (30 cm × 30 cm × 30 cm for rats; 20 cm × 20 cm × 20 cm for mice) and a smaller neutral compartment (15 cm × 10 cm × 30 cm for rats; 8 cm × 8 cm × 20 cm for mice). Each of the compartments was characterized by distinct visual, tactile, and olfactory stimuli. The experimental process consisted of three distinct sessions: a preconditioning session, a conditioning session, and a postconditioning session.

The formalin-induced conditioned place avoidance task required 5 days. Days 1 and 2 were preconditioning days. The animals were allowed to explore the two conditioning compartments freely for 10 min. Analysis software (Ethovision XT, Noldus, The Netherlands) was applied to record and analyze the movement path and the time spent in each of the compartments in a blinded manner. Animals that spent more than 450 s on one side on day 1 and more than 450 s on the other side on day 2 or that spent more than 80% on one side on day 2 (approximately 1% of all animals) were eliminated from the subsequent experiments. Days 3 and 4 were conditioning days. The rats received no treatment on day 3 and were limited to the conditioning compartment for 45 min. On day 4, the rats were given a unilateral intraplantar injection of formalin (5%, 50 μl) or normal saline (control) and then restrained in another conditioning compartment for 45 min. The mice received no treatment in the morning, and in the afternoon, after at least 4.5 h, the animals were given formalin (2.5% 10 μl)

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or normal saline as a control. The same procedures were repeated on days 3 and 4. Day 5 was the postconditioning day. The procedure was the same as that on days 1 and 2.

The estrogen receptor agonist–induced conditioned place avoidance task required 9 days. The preconditioning (days 1 to 2) and postconditioning (day 9) procedures were the same as those used in the formalin-induced conditioned place avoidance task. Days 3 to 8 were conditioning days. On day 3, the animals received no treatment and were confined to a conditioning compartment for 45 min. On day 4, the animals received bilateral microinjections of DPN (0.02 ng/μl, 0.5 μl/hemisphere), G1 (0.2 μg/μl, 0.5 μl/hemisphere), or vehicle (10% dimethyl sulfoxide, 0.5 μl/hemisphere) into the rostral anterior cingulate cortex and were then restrained in another conditioning compartment for 45 min. The same procedures performed on days 3 and 4 were repeated on days 5 to 8. The relative avoidance score was defined as the percentage of the difference in time spent in the treatment-paired compartment between the preconditioning test and postconditioning test relative to the time spent in the treatment-paired compartment in the preconditioning test.

The place escape/avoidance paradigm, a behavioral test that quantifies the level of aversion in a neuropathic pain-like model, was conducted as described previously.[23] Individual rats were placed in a 50 cm × 30 cm × 30 cm chamber on top of a raised mesh floor, one half of which was painted white (light area) and the other half of which was painted black (dark area). The animals were allowed to move unrestricted throughout the test chamber. A suprathreshold mechanical stimulus (60 g, von Frey filament) was applied to the plantar surface of the hind paws at 15-s intervals throughout the 30-min test period. The mechanical stimulus was applied to the affected paw (ipsilateral to the spared nerve injury) while the animal was located in the dark area, and the naive paw (contralateral to the spared nerve injury) was stimulated while the animal was in the light area. This test assesses whether a noxious stimulation is sufficiently bothersome for the animal to escape/avoid the preferred dark area and uses the amount of time spent on the nonpreferred light side to quantify the level of pain-related emotion.[24]

Formalin Nociceptive Test

Formalin (5%, 50 μl) was intraplantarly injected unilaterally into the hind paws of rats, and then the time spent lifting and licking the affected paw during each 5-min interval for 45 min after injection was recorded with a video recorder. The formalin nociceptive score was calculated using the following formula: formalin nociceptive score = [the time spent lifting the affected paw + 2 × (the time spent licking the affected paw licking)]/300.

Hargreaves Test and von Frey Test

Thermal hyperalgesia was assessed by measuring the paw withdrawal latency in response to a radiant heat source using the Hargreaves test. Briefly, the rats were placed individually in Plexiglas chambers on an elevated glass platform, under which a radiant heat source (IITC Life Science) was applied to the glabrous surface of the paw through the glass plate. The heat source was turned off when the rat lifted its foot, allowing the measurement of the paw withdrawal latency. A 20-s cutoff was used to prevent tissue damage in the absence of a response.

Mechanical allodynia was assessed by measuring the paw withdrawal threshold in response to a calibrated series of von Frey hairs (Stoelting). The rats were placed individually in a cage with a wire mesh bottom. A series of calibrated von Frey hairs was applied to the plantar surface of the hind paw in ascending order (2, 4, 6, 8, 10, 15, and 26 g) with sufficient force to bend the hair for 2 s or until paw withdrawal. A withdrawal response was considered valid only if the hind paw was completely removed from the customized platform. Each hair was applied five times, and the minimum value that caused at least three responses was recorded as the paw withdrawal threshold.

RNA Isolation and Real-time Polymerase Chain Reaction

Rostral anterior cingulate cortex tissues from adult male and female (gonadally intact and randomly cycling) rats were homogenized in TRIzol reagent (Invitrogen). Total RNA was extracted following the manufacturer's protocol. Primer sequences for estrogen receptor-α, estrogen receptor-β, and G protein–coupled estrogen receptor-1 were acquired from the National Center for Biotechnology Information online database. All polymerase chain reactions were performed using the polymerase chain reaction Master Mix reagents kit (Invitrogen Life Technologies) on a Promega Moloney murine leukemia virus sequence detection system (Promega Life Technologies). The specific polymerase chain reaction amplification products were detected with a fluorescent double-stranded DNA-binding dye. Each sample was run at least in triplicate, Ct values were averaged, and all of the samples with a coefficient of variation of the Ct value higher than 1% were retested. β-Actin was used as the reference gene to normalize the expression levels. The relative gene expression level was computed from Ct value of the target and β-actin using the following formula: messenger RNA (mRNA) relative expression $=2^{-(\text{Ct of target} - \text{Ct of } \beta\text{-actin})}$.

Immunohistochemistry

The animals were deeply anesthetized and transcardially perfused with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and postfixed in the same fixative for 8 to 12 h at 4°C and then dehydrated in gradient (10 to 30%) sucrose for 24 to 48 h at 4°C. Coronal sections (30 μm) were cut with a cryostat microtome (Leica CM 1950, Germany) and processed for immunohistochemistry. The sections were blocked with 10% normal donkey serum

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in TNB buffer (0.1 M Tris-HCl, 0.15 M NaCl, and 0.5% Blocking reagent, pH 7.5) for 1 h at room temperature and incubated overnight at 4°C with individual or a mixture of primary antibodies: rabbit anti–estrogen receptor-α (1:50, Santa Cruz, USA), rabbit anti–estrogen receptor-β (1:100, Santa Cruz), rabbit anti–G protein–coupled estrogen receptor-1 (1:200, Abcam, United Kingdom), mouse anti–βIII-tubulin (1:1,000, Millipore, USA), and chicken anti-YFP/GFP (1:500, Aves, USA). Corresponding Alexa Fluor 488– or 546–conjugated secondary antibodies (1:200, Invitrogen, USA) and biotinylated secondary antibodies (Vector Laboratories, USA) were applied for 2 h at 4°C. The sections were washed and incubated with streptavidin–horseradish peroxidase for 30 min and then incubated with fluorophore tyramine working solution. All sections were coverslipped with Fluoromount aqueous mounting medium (Sigma) and then observed and analyzed with a confocal laser-scanning microscope (Olympus FV1000, Japan). The specificity of immunostaining was verified by omitting the primary antibodies, and the immunostaining signal disappeared after the primary antibodies were omitted. The specificity of the primary antibodies was verified by a preabsorption experiment involving incubation with a mixture of estrogen receptor-α, estrogen receptor-β, and G protein–coupled estrogen receptor-1 primary antibodies and a corresponding blocking peptide (peptide:primary antibody = 5:1) overnight.

Slice Preparation and Whole-cell Recordings

Brain slices were prepared as described previously.[4] For Western blot experiments, the slices were treated with different agents: 17β-estradiol (1 μM), PPT (1 μM), DPN (100 nM), or G1 (100 nM) was added to artificial cerebrospinal fluid for 10 min. PHTPP (1 μM) or G15 (1 μM) was added to artificial cerebrospinal fluid for 20 min before and during treatment with 17β-estradiol. Subsequently, the rostral anterior cingulate cortex was dissected on ice using a surgical blade, rapidly frozen in liquid nitrogen, and then stored at −80°C for further processing. For electrophysiologic recordings, a single slice was transferred to a recording chamber and continuously perfused with recording solution at a rate of 5 ml/min at room temperature.

Whole-cell patch-clamp recordings were performed as described previously.[4] In brief, evoked excitatory postsynaptic currents were induced by repetitive 0.05-Hz stimuli that were delivered by a bipolar tungsten stimulating electrode placed in layer V of the rostral anterior cingulate cortex. Recording electrodes were filled with an intracellular solution containing 150 mM gluconate, 8 mM NaCl, 0.4 mM ethylene glycol tetraacetic acid, 2 mM Mg adenosine triphosphate, 0.3 mM Na_3 guanosine triphosphate, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, with the pH adjusted to 7.2 and osmolality adjusted to 300 mOsmol/l. Pyramidal-shaped neurons in layers II/III were easily identified by their shape and electrophysiologic properties. The NMDA-mediated component of excitatory postsynaptic currents was recorded in low-Mg^{2+} (0.1 mM) and high-Ca^{2+} (3.8 mM) artificial cerebrospinal fluid containing 6-cyano-7-nitroquinoxaline-2,3-dione (20 μM) and bicuculline methiodide (10 μM), and the membrane potential was held at −40 mV. The drugs applied in the perfusate were as follows: 100 nM DPN, 100 nM G1, 1 μM PPT, 100 nM 17β-estradiol, 5 μM Rp-cAMP, 100 nM wortmannin, and 1 μM tetrodotoxin. Tetrodotoxin was included in the perfusate to prevent polysynaptic phenomena. The data were collected with pClamp 10.6 software and were analyzed using Clampfit 10.6.

Isolation of the Subcellular Fraction and Western Blotting

The animals were sacrificed, and the brains were quickly removed. The rostral anterior cingulate cortex was dissected on ice using a rat or mouse brain matrix (Stoelting, USA) and then separated into the left and right halves from the sagittal midline. Cytoplasmic and/or membrane and nuclear proteins were extracted using a compartment protein extraction kit (Chemicon, 2145, USA) according to the manufacturer's protocol. The protein concentrations of the three fractions were measured. The proteins were aliquoted, labeled properly, and stored at −80°C. The synaptosomal membrane fraction was extracted as previously described[25,26] with minor modifications. Rostral anterior cingulate cortex tissues were homogenized with homogenization buffer A (Roche, Switzerland). Some of each homogenate was preserved as the total protein homogenate (H). The remaining homogenate was centrifuged at 1,000*g* for 10 min at 4°C to obtain the insoluble precipitate (P1) containing nuclei and large debris. The supernatant (S1) was again centrifuged at 10,000*g* for 30 min to generate a crude synaptosomal fraction (P2) and supernatant (S2). The crude synaptosomal membrane precipitate (P2) was resuspended in buffer A and centrifuged at 25,000*g* for 30 min to generate the synaptosomal membrane fraction (LP1) and supernatant (LS1). LP1 and H were separately resuspended in ice-cold homogenization buffer B (Roche) and then centrifuged at 15,000*g* for 5 min to obtain supernatant for Western blot analysis.

Equal amounts of protein (~20 μg) were loaded and separated on 10% Tris–Tricine SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% nonfat milk for 2 h and incubated overnight at 4°C with rabbit anti–estrogen receptor-α (1:1,000, Upstate, USA), rabbit anti–estrogen receptor-β (1:500, Upstate), mouse anti–G protein–coupled estrogen receptor-1 (1:200, Abcam, United Kingdom), mouse anti-transferrin receptor (1:1,000, Invitrogen, USA), rabbit anti-CREB (1:3,000, Upstate USA), mouse anti-GluN2B (1:2,000; Neuromab, USA), rabbit anti-GluN1 (1:2,000; Sigma, USA), mouse anti-SNAP25 (1:10,000; Synaptic Systems, Germany), rabbit anti–phospho-AKT (Ser473; 1:2,000, Cell Signaling, USA), and

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rabbit anti–phosphoprotein kinase A C (Thr197; 1:8,000, Cell Signaling) primary antibodies and then incubated with horseradish peroxidase–conjugated secondary antibodies (1:4,000, Pierce, USA) for 2 h at 4°C. A glyceraldehyde-3-phosphate dehydrogenase antibody (1:20,000, Aksomics, China) was probed as a loading control. The signals were finally visualized using enhanced chemiluminescence (Pierce) and captured by a ChemiDoc XRS system (Bio-Rad, USA). All Western blot analyses were performed at least three times, and consistent results were obtained. A Bio-Rad image analysis system was used to measure the integrated optic density of the specific bands.

Statistical Analysis

The data are presented as the means ± SD. No statistical power calculation was conducted before the study. The sample sizes were based on our previous knowledge and experience with this design. There were no missing data except for that of three rats that were excluded from the experiments because of their preference (more than 80%) for one compartment in the preconditioning phase of the conditioned place avoidance experiment. All data from the different groups were verified for normality and homogeneity of variance using Kolmogorov–Smirnov and Brown–Forsythe tests before analysis. For the conditioned place avoidance experiments, the time spent in the treatment-paired compartment on the preconditioning day *versus* the postconditioning day was compared using a paired *t* test. The differences in avoidance scores, protein expression (Western blot analysis), and mRNA levels (real-time polymerase chain reaction) among the drug-treated groups were compared using Student's *t* test when comparing two groups or one-way ANOVA followed by *post hoc* Dunnett's test when comparing more than two groups. For formalin-induced nociceptive behavior, the data from the place escape/avoidance paradigm test and NMDA-mediated long-term potentiation were analyzed using two-way repeated-measures ANOVA followed by *post hoc* Bonferroni multiple comparison test. No additional data were excluded from the statistical analyses because of outlier status. All analyses were two-tailed, and a *P* value less than 0.05 was considered statistically significant. The statistical analyses were performed using GraphPad Prism 7.0 software.

Results

Estrogen Receptor-α, Estrogen Receptor-β, and G Protein–coupled Estrogen Receptor-1 Are Abundantly Expressed in the Rostral Anterior Cingulate Cortex, Especially in Excitatory Pyramidal Neurons

All three subtypes of estrogen receptors were abundantly expressed in the rostral anterior cingulate cortex (fig. 1A). The mRNAs of the three estrogen receptors were also detected in the rostral anterior cingulate cortex in both male and female rats without sex differences (Supplemental Digital Content, fig. S1A, http://links.lww.com/ALN/C350). Double immunofluorescence showed that nearly all three estrogen receptors were colocalized with the neuronal marker βIII-tubulin (fig. 1B). Subcellular fractionation analysis revealed that estrogen receptor-α and estrogen receptor-β were detected in the cell membrane, cytoplasm, and nucleus in the rostral anterior cingulate cortex, whereas G protein–coupled estrogen receptor 1 mostly existed in the membrane and cytoplasm (Supplemental Digital Content, fig. S1B, http://links.lww.com/ALN/C350). In addition to neuronal soma, estrogen receptor-α and estrogen receptor-β were enriched in dendrites because both estrogen receptor-α and estrogen receptor-β were colocalized with the neuronal dendrite marker MAP-2 (Supplemental Digital Content, fig. S1C, http://links.lww.com/ALN/C350).

We further examined the expression of estrogen receptor-α, estrogen receptor-β and G protein–coupled estrogen receptor-1 in CaMK2α::EYFP mice. The estrogen receptors were heavily colocalized with CaMK2α-EYFP in the rostral anterior cingulate cortex, indicating that most rostral anterior cingulate cortex excitatory pyramidal neurons expressed estrogen receptors (fig. 1, C to F).

Estrogen Receptor-β and G Protein–coupled Estrogen Receptor-1 but Not Estrogen Receptor-α in the Rostral Anterior Cingulate Cortex Contribute to the Induction of Pain-related Aversion

As mentioned above, our previous study demonstrated that brain-derived estrogen drives the emotional component of pain without sex specificity.[4] Additionally, as shown in Supplemental Digital Content, figure S1A (http://links.lww.com/ALN/C350), no difference in the mRNA levels of the estrogen receptors in the rostral anterior cingulate cortex was found between males and females. Thus, in the present study, male rats and mice were used in subsequent experiments to exclude the influence of the estrous cycle of females.

Formalin-induced conditioned place avoidance, a well designed model for investigating pain-related aversion in rodents,[22,27] was used to evaluate affective responses to inflammatory pain. The rats that received a unilateral intraplantar injection of 5% formalin spent obviously less time in the compartment paired with treatment on the postconditioning day than on the preconditioning day (mean ± SD: postconditioning: 193 ± 91 s *vs.* preconditioning: 381 ± 45 s; two-tailed paired *t* test, $t_{(7)}$ =5.24, P = 0.001). Control animals that received an intraplantar injection of normal saline (mean ± SD: postconditioning: 308 ± 73 s *vs.* preconditioning: 326 ± 34 s; two-tailed paired *t* test, $t_{(7)}$ =5.24, P = 0.26) did not exhibit conditioned place avoidance (fig. 2, A to C). The relative avoidance score of the formalin-treated group (the percentage of the difference of time spent in the treatment-paired compartment between the preconditioning test and postconditioning test relative to the time spent in the treatment-paired compartment in the preconditioning test) was statistically higher than that of the saline-treated group (mean ± SD: formalin-treated group: 48.8 ± 23.5%

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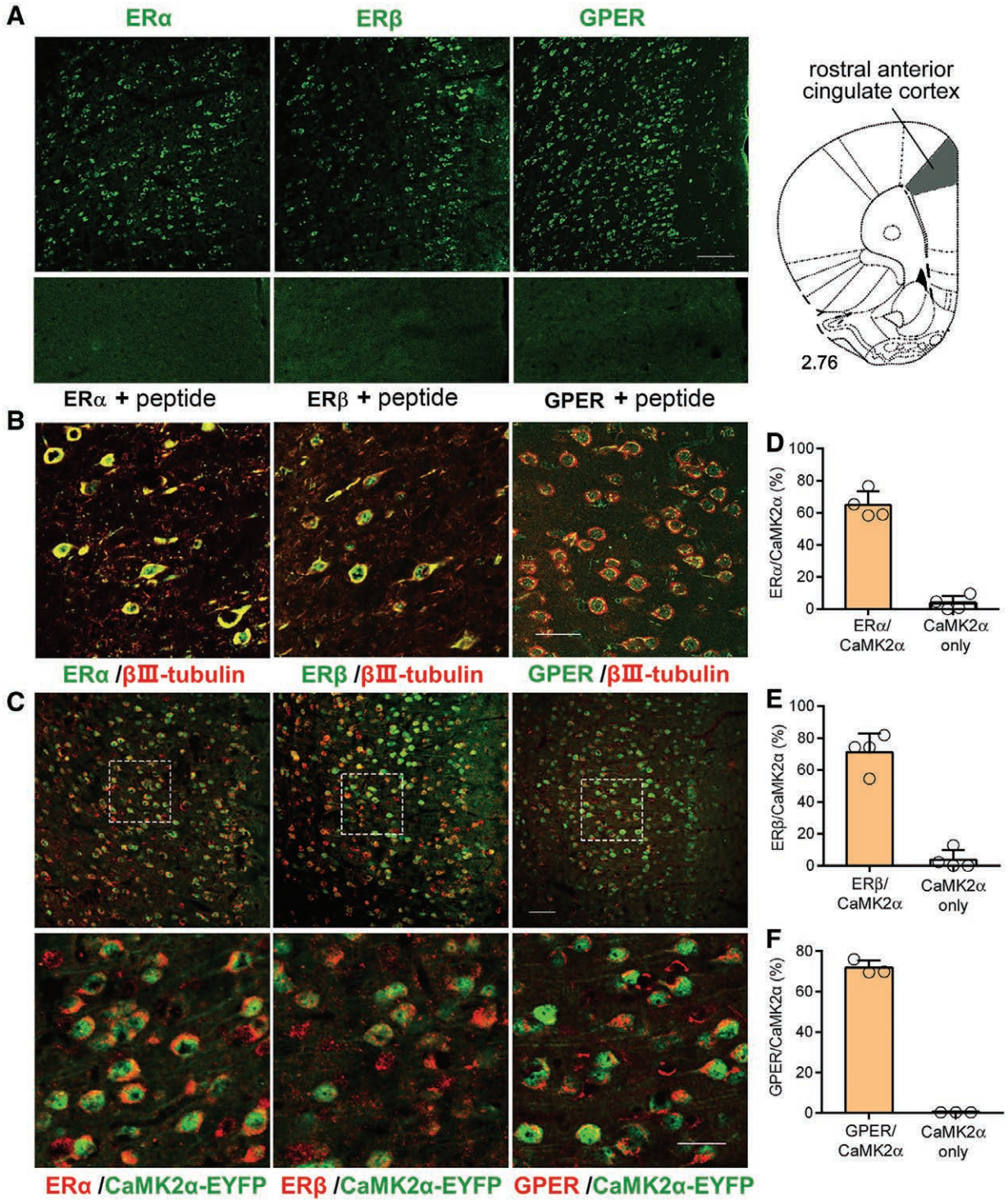

Fig. 1. Distribution of estrogen receptors in the rostral anterior cingulate cortex. (*A*, *left*) Sections showing estrogen receptor-α (ERα), estrogen receptor-β (ERβ), and G protein–coupled estrogen receptor-1 (GPER) immunoreactivity in the rat rostral anterior cingulate cortex. (*A*, *right*) Schematic drawing showing the anterior cingulate cortex and the surrounding area. *Scale bar*, 100 μm. (*B*) Double staining showing the colocalization of estrogen receptor-α, estrogen receptor-β, and G protein–coupled estrogen receptor-1 (*green*) with the neuronal marker βIII-tubulin (*red*) in the rat rostral anterior cingulate cortex. *Scale bar*, 40 μm. (*C*) The colocalization of estrogen receptor-α, estrogen receptor-β, and G protein–coupled estrogen receptor-1 (*red*) with CaMK2α-EYFP (*green*) in the rostral anterior cingulate cortex in CaMK2α::EYFP mice. *Upper scale bar*, 60 μm; *lower scale bar*, 30 μm. (*D–F*) Quantification of CaMK2α-EYFP–positive neurons expressing estrogen receptor-α (*D*), estrogen receptor-β (*E*), and G protein–coupled estrogen receptor-1 (*F*) in the rostral anterior cingulate cortex.

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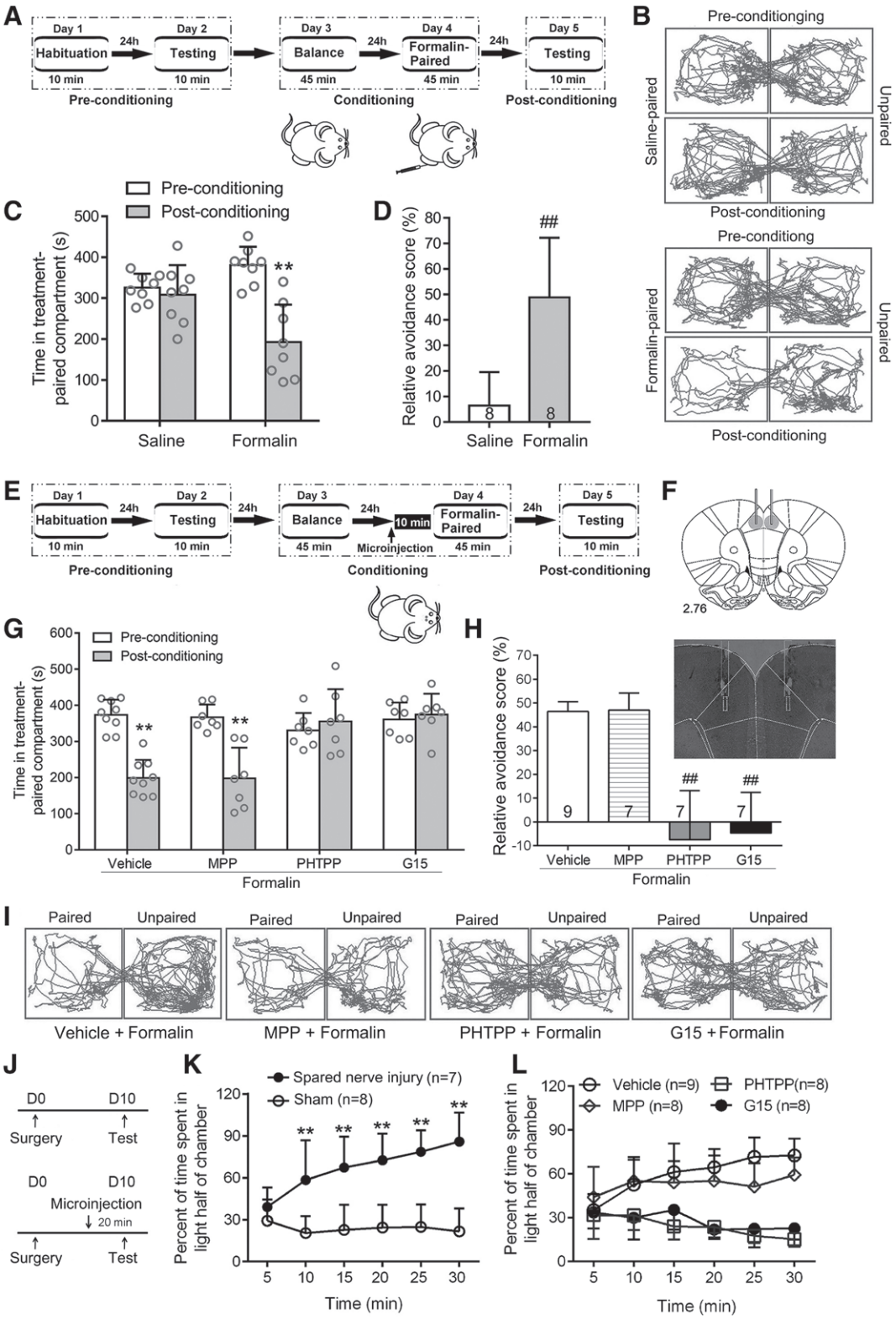

Fig. 2. Estrogen receptor-β and G protein–coupled estrogen receptor-1 but not estrogen receptor-α contribute to formalin-induced conditioned place avoidance and place escape/avoidance paradigm acquisition. (*A*) Schematic of the protocol for the formalin-induced conditioned place avoidance task in rats. (*B*) Example tracks of the rats before and after conditioning. (*C*, *D*) Successful establishment of formalin-induced

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vs. saline-treated group: 6.5 ± 13.1%; two-tailed Student's *t* test, $t_{(14)}$ =5.24, $P = 0.001$; fig. 2D).

To address whether estrogen receptor-α, estrogen receptor-β, and G protein–coupled estrogen receptor-1 contribute to pain-related aversion, the G protein–coupled estrogen receptor-1 antagonist G15 (1.8 μg/μl, 0.5 μl/hemisphere), the estrogen receptor-β antagonist PHTPP (1 ng/μl, 0.5 μl/hemisphere), or the estrogen receptor-α antagonist MPP (1 ng/μl, 0.5 μl/hemisphere) was bilaterally microinjected into the rostral anterior cingulate cortex 10 min before formalin-paired conditioning (fig. 2, E and F). The acquisition of formalin-induced conditioned place avoidance was blocked by PHTPP or G15 but not by MPP; the relative avoidance scores were significantly different among the treated groups (mean ± SD: MPP: 47.0 ± 18.9%, −7.4 ± 20.6%, and G15: −4.6 ± 17.0% *vs.* vehicle: 46.5 ± 12.2%; one-way ANOVA, $F_{(3,26)} = 23.68$, $P < 0.0001$; fig. 2, G to I). These data suggest that estrogen receptor-β or G protein–coupled estrogen receptor-1 but not estrogen receptor-α in the rostral anterior cingulate cortex is required for the acquisition of formalin-induced conditioned place avoidance. To further confirm the effects of estrogen receptors on pain-related negative emotion, the pain-evoked place escape/avoidance paradigm was used to evaluate emotional responses in a neuropathic pain model. As shown in figure 2 (J and K), spared nerve injury rats spent markedly more time on the light side of the chamber, suggesting that these animals were willing to avoid a preferred area (dark side) to escape stimulation of the affected paw. Control animals preferred to remain in the dark and tended to avoid the light side of the chamber (two-way repeated-measures ANOVA, main effect of group: $F_{(1,13)}$=127.40, $P < 0.0001$; group × time interaction: $F_{(5,78)} = 3.92$, $P = 0.003$; fig. 2K). The nerve injury rats that received bilateral injections of PHTPP or G15 into the rostral anterior cingulate cortex spent obviously less time in the light side of the chamber than rats that received vehicle injection, whereas injections of MPP into the rostral anterior cingulate cortex had no effect on place escape/avoidance (two-way ANOVA, main effect of group: $F_{(3,29)} = 49.72$, $P < 0.0001$; group × time interaction: $F_{(15,145)} = 5.43$, $P < 0.0001$; fig. 2L).

To determine whether estrogen receptor-β or G protein–coupled estrogen receptor-1 in the rostral anterior cingulate cortex is specific for pain-related aversion rather than reducing formalin-induced nociceptive sensitivity, the effects of PHTPP and G15 on formalin-induced spontaneous nociceptive responses were examined. In general, the intraplantar injection of dilute formalin elicited characteristic biphasic nociceptive responses, including lifting, licking, and biting, in rodents. The microinjection of PHTPP or G15 into the rostral anterior cingulate cortex did not affect formalin-induced nociceptive responses (fig. 3).

As shown in figure 1, estrogen receptors are predominantly expressed in CaMK2α-positive excitatory pyramidal neurons in the rostral anterior cingulate cortex. To further determine the effects of estrogen receptor-α and estrogen receptor-β in rostral anterior cingulate cortex excitatory neurons on pain-related aversion, an adeno-associated virus expressing estrogen receptor-α–short-hairpin RNA or estrogen receptor-β–short-hairpin RNA driven by CaMK2α was injected into the rostral anterior cingulate cortex to knock down estrogen receptor-α or estrogen receptor-β, respectively, in rostral anterior cingulate cortex excitatory pyramidal neurons. Western blot analyses showed that estrogen receptor-α and estrogen receptor-β protein levels were successfully suppressed by estrogen receptor-α– and estrogen receptor-β–short-hairpin RNA, respectively, 2 to 3 weeks after being injected into the rostral anterior cingulate cortex injection without cross-effects (fig. 4, A

Fig. 2. (*Continued*). conditioned place avoidance, as indicated by the time spent in the treatment (intraplantar injection of normal saline or formalin)–paired compartment in the preconditioning and postconditioning tests (*C*) and the relative avoidance scores (*D*). $^{**}P < 0.01$ *versus* the preconditioning test (two-tailed paired *t* test); $^{\#\#}P < 0.01$ *versus* normal saline (two-tailed Student's *t* test); n = 8 and 8. (*E*) Schematic of the protocol for experiments *G* to *I*. (*F*) Schematic of bilateral injections of estrogen receptor antagonists into the rostral anterior cingulate cortex and a photomicrograph of a coronal section showing the cannula placement in the bilateral rostral anterior cingulate cortex. (*G*, *H*) Microinjections of the estrogen receptor-β antagonist 4-(2-phenyl-5,7-bis [trifluoromethyl] pyrazolo [1,5-a] pyrimidin-3-yl) phenol (PHTPP; 1 ng/μl, 0.5 μl/hemisphere) or the G protein–coupled estrogen receptor-1 antagonist (3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta [c] quinolone (G15; 1.8 μg/μl, 0.5 μl/hemisphere) into the rostral anterior cingulate cortex significantly blocked formalin-induced conditioned place avoidance, whereas the estrogen receptor-α antagonist 1,3-Bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole dihydrochloride (MPP, 1 ng/μl, 0.5 μl/hemisphere) had no effect. $^{**}P < 0.01$ *versus* the preconditioning test (two-tailed paired *t* test); $^{\#\#}P < 0.01$ *versus* vehicle (10% dimethyl sulfoxide; one-way ANOVA followed by *post hoc* Dunnett's test); n = 9, 7, 7, and 7. (*I*) Example tracks of the rats after conditioning in the post-conditioning day. (*J*) Schematic of the protocol for experiments *K* and *L*. (*K*) Spared nerve injury rats spent significantly more time on the light side of the chamber in the place escape/avoidance paradigm, indicating that these animals were willing to avoid a preferred area (dark area) to escape stimulation of the affected paw. Control animals preferred to remain in the dark and tended to avoid the light side of the chamber. $^{**}P < 0.01$ *versus* the sham control (two-way repeated-measures ANOVA followed by *post hoc* Bonferroni multiple comparison test); n = 7 and 8. (*L*) Microinjections of the estrogen receptor-β antagonist PHTPP (1 ng/μl, 0.5 μl/hemisphere) or the G protein–coupled estrogen receptor-1 antagonist G15 (1.8 μg/μl, 0.5 μl/hemisphere) into the rostral anterior cingulate cortex significantly blocked place escape/avoidance, whereas the estrogen receptor-α antagonist MPP (1 ng/μl, 0.5 μl/hemisphere) had no effect. $^{**}P < 0.01$ *versus* vehicle (two-way repeated-measures ANOVA followed by *post hoc* Bonferroni multiple comparison test); n = 9, 8, 8, and 8.

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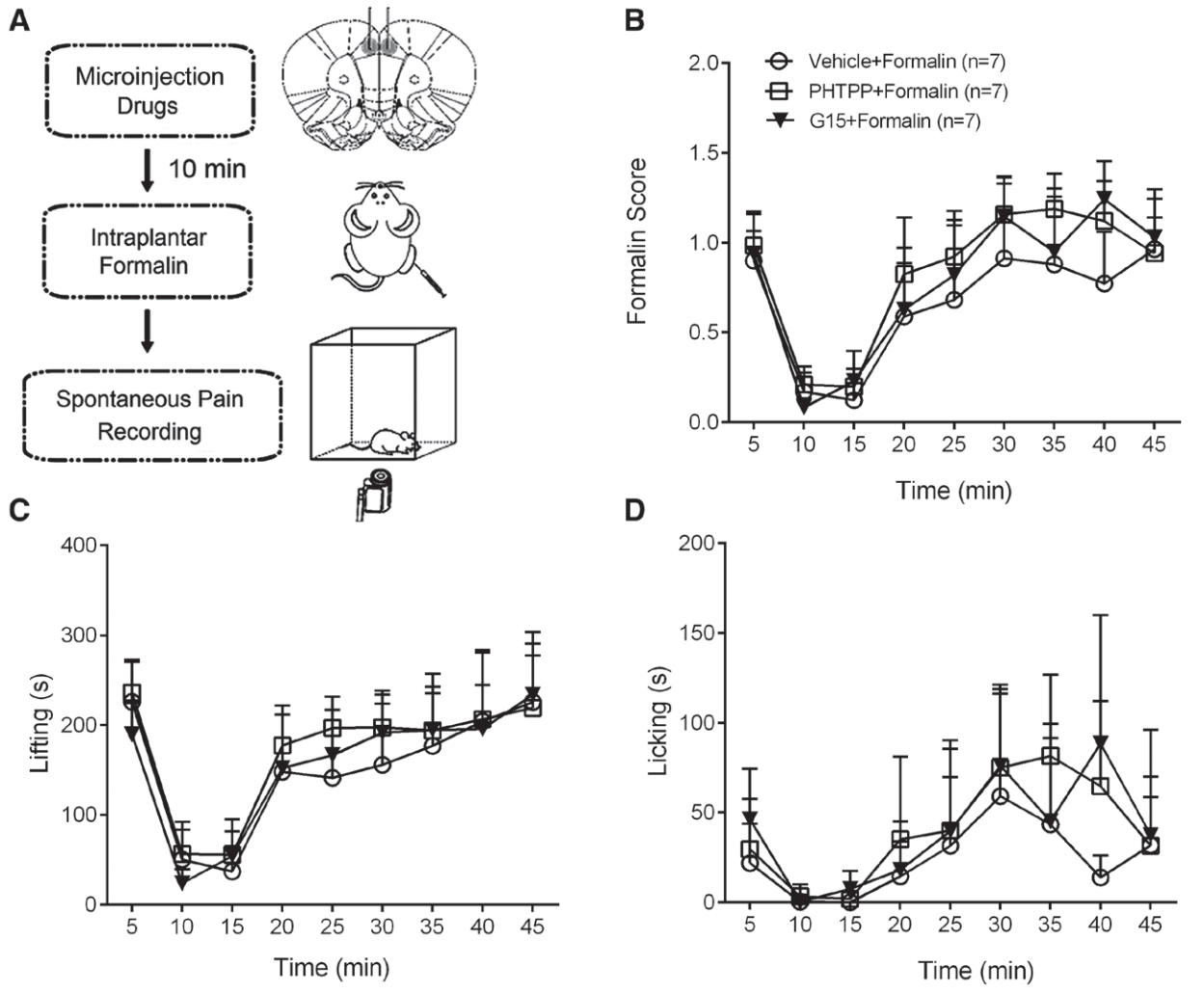

Fig. 3. Effects of blocking estrogen receptor-β and G protein–coupled estrogen receptor-1 in the rostral anterior cingulate cortex on formalin-induced spontaneous nociceptive responses. (*A*) Schematic of the protocol for measuring formalin-induced nociceptive responses. (*B–D*) Intraplantar injection of 5% formalin induced a two-phase nociceptive response in rats. Microinjections of the estrogen receptor-β antagonist 4-(2-phenyl-5,7-bis [trifluoromethyl] pyrazolo [1,5-a] pyrimidin-3-yl) phenol (PHTPP; 1 ng/μl, 0.5 μl/hemisphere) or the G protein–coupled estrogen receptor-1 antagonist (3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta [c] quinolone (G15; 1.8 μg/μl, 0.5 μl/hemisphere) into the rostral anterior cingulate cortex had no effect on spontaneous pain-like agitation, including lifting and licking (two-way repeated-measures ANOVA); n = 7, 7, and 7.

to D). Similar to the rats, the mice that received a unilateral intraplantar injection of 2.5% formalin spent less time in the paired conditioning compartment than those that received normal saline (Supplemental Digital Content, fig. S2, http://links.lww.com/ALN/C350). The mice that received bilateral injections of AAV9–CaMK2α–shERβ–EGFP into the rostral anterior cingulate cortex failed to acquire formalin-induced conditioned place avoidance, whereas AAV9–CaMK2α–shERα–EGFP and AAV9–CaMK2α–shControl–EGFP had no effect on formalin-induced conditioned place avoidance (fig. 4, E and F). The relative avoidance scores among the three groups were statistically different (mean ± SD: shERα: 75.0 ± 19.6%, and estrogen receptor-β–short-hairpin RNA: 18.4 ± 36.9% *vs.* short-hairpin control: 92.4 ± 34.9%; one-way ANOVA, $F_{(2,23)} = 13.37$, $P < 0.0001$; fig. 4G). These results further confirmed that estrogen receptor-β but not estrogen receptor-α in rostral anterior cingulate cortex neurons is necessary for pain-related aversion.

Estrogen Receptor-β and G Protein–coupled Estrogen Receptor-1 but Not Estrogen Receptor-α Are Involved in NMDA Receptor–mediated Long-term Potentiation in Rostral Anterior Cingulate Cortex Pyramidal Neurons

Our previous studies have shown that the NMDA receptors are critically involved in pain-related aversion,[22,28,29] and

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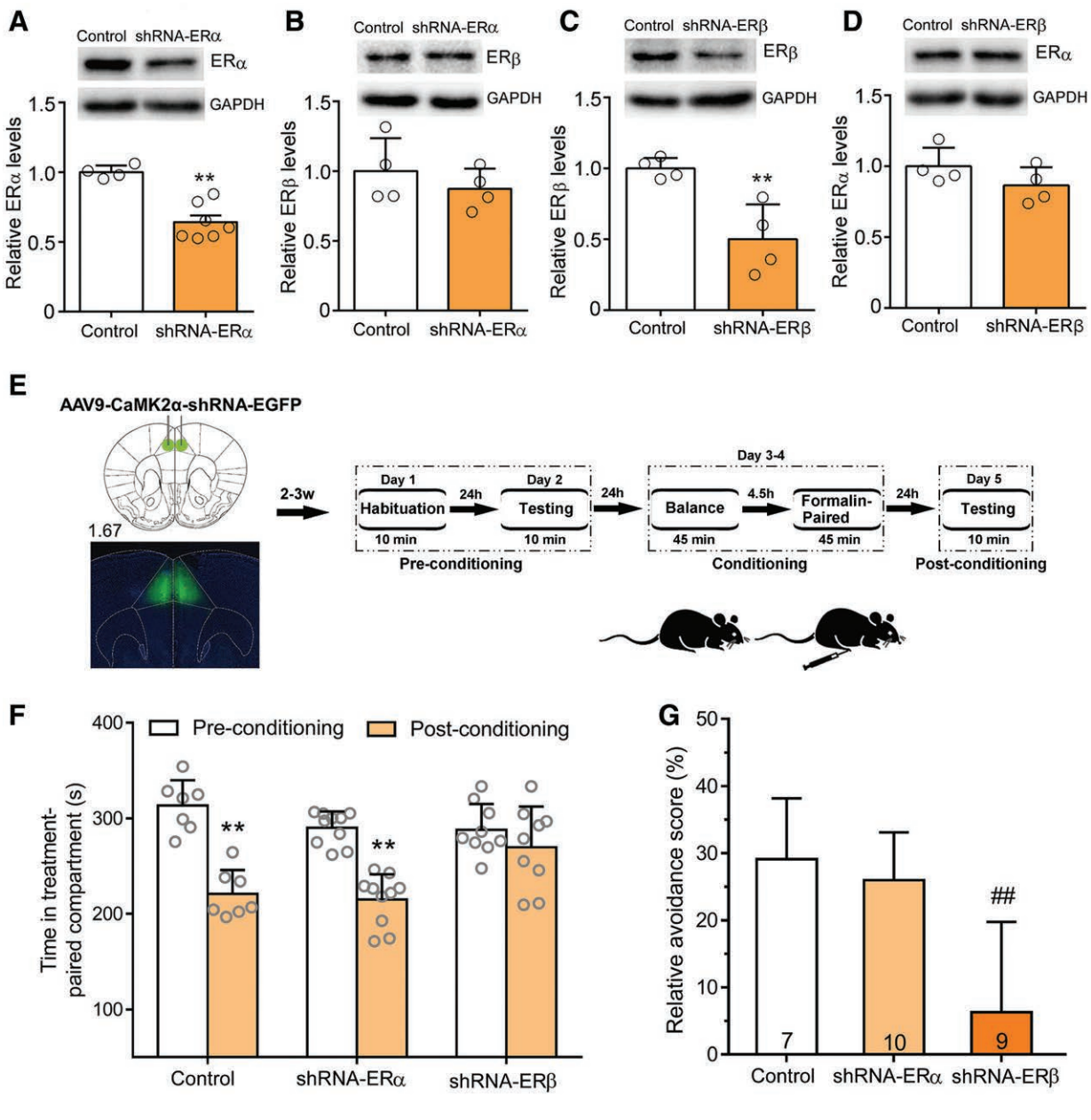

Fig.4. Knockdown of estrogen receptor-β (ERβ) but not estrogen receptor-α in rostral anterior cingulate cortex excitatory pyramidal neurons prevents formalin-induced conditioned place avoidance acquisition. (*A*, *B*) The administration of estrogen receptor-α short-hairpin RNA (shRNA-ERα) into the rostral anterior cingulate cortex robustly downregulated estrogen receptor-α (ERα) levels in the rostral anterior cingulate cortex but had no effect on estrogen receptor-β expression. (*C*, *D*) Estrogen receptor-β short-hairpin RNA (shRNA-ERβ) reduces estrogen receptor-α levels but did not affect estrogen receptor-α expression. $^{**}P < 0.01$ *versus* control short-hairpin RNA (two-tailed Student's *t* test); n = 4 and 4. (*E*) A schematic and photomicrograph of coronal sections showing AAV9–CaMK2α–short-hairpin RNA–EGFP injection into the bilateral rostral anterior cingulate cortex and the experimental protocol. (*F*, *G*) Knockdown of estrogen receptor-β by short-hairpin RNA (shRNA-ERβ) in the rostral anterior cingulate cortex prevented formalin-induced conditioned place avoidance induction. The administration of estrogen receptor-α short-hairpin RNA (shRNA-ERα) or nontargeting control short-hairpin RNA (control) into the rostral anterior cingulate cortex did not affect formalin-induced conditioned place avoidance. $^{**}P < 0.01$ *versus* the preconditioning test (two-tailed paired *t* test); $^{\#\#}P < 0.01$ *versus* control short-hairpin RNA (control, one-way ANOVA followed by *post hoc* Dunnett's test); n = 7, 10, and 9. shRNA, short-hairpin RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFP, enhanced green fluorescent protein.

17β-estradiol in the rostral anterior cingulate cortex rapidly enhances NMDA receptor–mediated synaptic transmission and plasticity, driving the emotional component of pain.[4] Whole-cell patch-clamp recordings were performed in

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pyramidal neurons in layers II/III of rostral anterior cingulate cortex slices. We identified pyramidal neurons based on the pyramidal shape of their soma and by injecting depolarized currents into neurons to induce action potentials.[30] Evoked excitatory postsynaptic currents were obtained by delivering focal electrical stimulation to layer V of the rostral anterior cingulate cortex. The NMDA receptor–mediated component of excitatory postsynaptic currents (NMDA-mediated excitatory postsynaptic currents) was recorded in artificial cerebrospinal fluid containing 6-cyano-7-nitroquinoxaline-2,3-dione (20 μM) and bicuculline methiodide (10 μM), and neurons were voltage clamped at −40 mV. Bath application of the estrogen receptor-β agonist DPN (100 nM) or the G protein–coupled estrogen receptor-1 agonist G1 (100 nM) for 6 min rapidly elicited long-lasting potentiation of NMDA-mediated excitatory postsynaptic currents, which was defined as the chemical long-term potentiation of NMDA-mediated excitatory postsynaptic currents (fig. 5, A and B). Consistent with what was observed in the formalin-induced conditioned place avoidance experiment, the estrogen receptor-α agonist PPT (1 μM) failed to elicit NMDA-mediated long-term potentiation (fig. 5C). However, exposure to 17β-estradiol (100 nM) after PPT application directly elicited NMDA-mediated long-term potentiation (fig. 5D), confirming that PPT does not have an effect on NMDA receptor–dependent synaptic plasticity. Moreover, bath application of DPN (100 nM), G1 (100 nM), or 17β-estradiol (1 μM) for 10 min produced a robust increase in the expression of the NMDA receptor subunits GluN1 and GluN2B in the synaptosomal membrane, but PPT (1 μM) treatment had no effect (Supplemental Digital Content, fig. S3, A and B, http://links.lww.com/ALN/C350). Additionally, 17β-estradiol exposure (slices) or formalin treatment (*in vivo*) augmented the expression of estrogen receptor-β and G protein–coupled estrogen receptor-1 but did not affect estrogen receptor-α expression in the synaptosomal membrane in the rostral anterior cingulate cortex (Supplemental Digital Content, fig. S3, C and D, http://links.lww.com/ALN/C350). Together with the results of our previous study, these results suggest that estrogen receptor-β and G protein–coupled estrogen receptor-1 might mediate the estrogen-driven emotional component of pain by facilitating NMDA receptor–dependent excitatory synaptic transmission and plasticity.

Activation of Estrogen Receptor-β or G Protein–coupled Estrogen Receptor-1 in the Rostral Anterior Cingulate Cortex Elicits Conditioned Place Avoidance *via* the Protein Kinase A or Protein Kinase B Pathway

A previous study demonstrated that excitatory amino acids in the rostral anterior cingulate cortex directly produce avoidance learning in the absence of noxious stimulation.[31] In addition, the present study showed that the activation of estrogen receptor-β or G protein–coupled estrogen receptor-1 rapidly elicits NMDA-mediated long-term potentiation. We therefore tested whether the activation of estrogen receptor-β or G protein–coupled estrogen receptor-1 *per se* is sufficient to elicit conditioned place avoidance. The estrogen receptor-β agonist DPN (0.02 ng/μl, 0.5 μl/hemisphere) or the G protein–coupled estrogen receptor-1 agonist G1 (0.2 μg/μl, 0.5 μl/hemisphere) was microinjected into the bilateral rostral anterior cingulate cortex in the absence of formalin stimulation (fig. 5E). Rats from both the DPN and G1 groups spent less time in the agonist-paired compartment than the vehicle-injected group (fig. 5, F and G). The relative avoidance scores of the DPN group and the G1 group was robustly higher than those of the vehicle group (mean ± SD: DPN group: 35.3 ± 9.5% and G1 group: 43.5 ± 22.8% *vs.* vehicle group: 0.4 ± 16.0%; one-way ANOVA, $F_{(2,21)} = 15.25$, $P < 0.0001$; fig. 5H). Interestingly, the injection of any of the three estrogen receptor agonists in the rostral anterior cingulate cortex failed to alter mechanical and thermal sensitivity (Supplemental Digital Content, fig. S4, http://links.lww.com/ALN/C350). These results strongly indicate that estrogen receptor-β and G protein–coupled estrogen receptor-1 in the rostral anterior cingulate cortex primarily mediate estrogen-dependent aversive learning.

We further detected the possible downstream signaling pathways that mediate the action of estrogen receptor-β or G protein–coupled estrogen receptor-1 in the rostral anterior cingulate cortex. Estrogen receptors have been found to rapidly activate protein kinase pathways, including the phosphoinositide 3-kinase/protein kinase B and cAMP/protein kinase A signaling pathways.[32,33] Rostral anterior cingulate cortex slices were treated with the following: vehicle, 17β-estradiol, 17β-estradiol plus the estrogen receptor-β inhibitor PHTPP or the 17β-estradiol plus the G protein–coupled estrogen receptor-1 inhibitor G15 (fig. 6A). Exposure to 17β-estradiol (1 μM) for 10 min induced a remarkable increase in the phosphorylated protein kinase A catalytic subunit and phosphorylated protein kinase B (fig. 6, B and C). Interestingly, the preincubation of rostral anterior cingulate cortex slices with PHTPP (1 μM) for 20 min suppressed the 17β-estradiol–induced upregulation of the phosphorylated protein kinase A catalytic subunit but not phosphorylated protein kinase B. In contrast, the 17β-estradiol–induced increase in phosphorylated protein kinase B but not the phosphorylated protein kinase A catalytic subunit was blocked by G15 (1 μM; one-way ANOVA, phosphorylated protein kinase A catalytic subunit: $F_{(3,19)} = 15.75$, $P < 0.0001$; phosphorylated protein kinase B: $F_{(3,19)} = 8.47$, $P = 0.001$). Additionally, we observed robust activation of protein kinase A and protein kinase B in the bilateral rostral anterior cingulate cortex after unilateral intraplantar formalin injection (fig. 6, D to F).

Moreover, microinjections of the protein kinase A inhibitor Rp-cAMP (1 μg/μl, 0.5 μl/hemisphere) or the protein kinase B inhibitor wortmannin (0.2 μg/μl, 0.5 μl/hemisphere) into the bilateral rostral anterior cingulate cortex robustly blocked formalin-induced conditioned place avoidance (fig. 6, G to I). The

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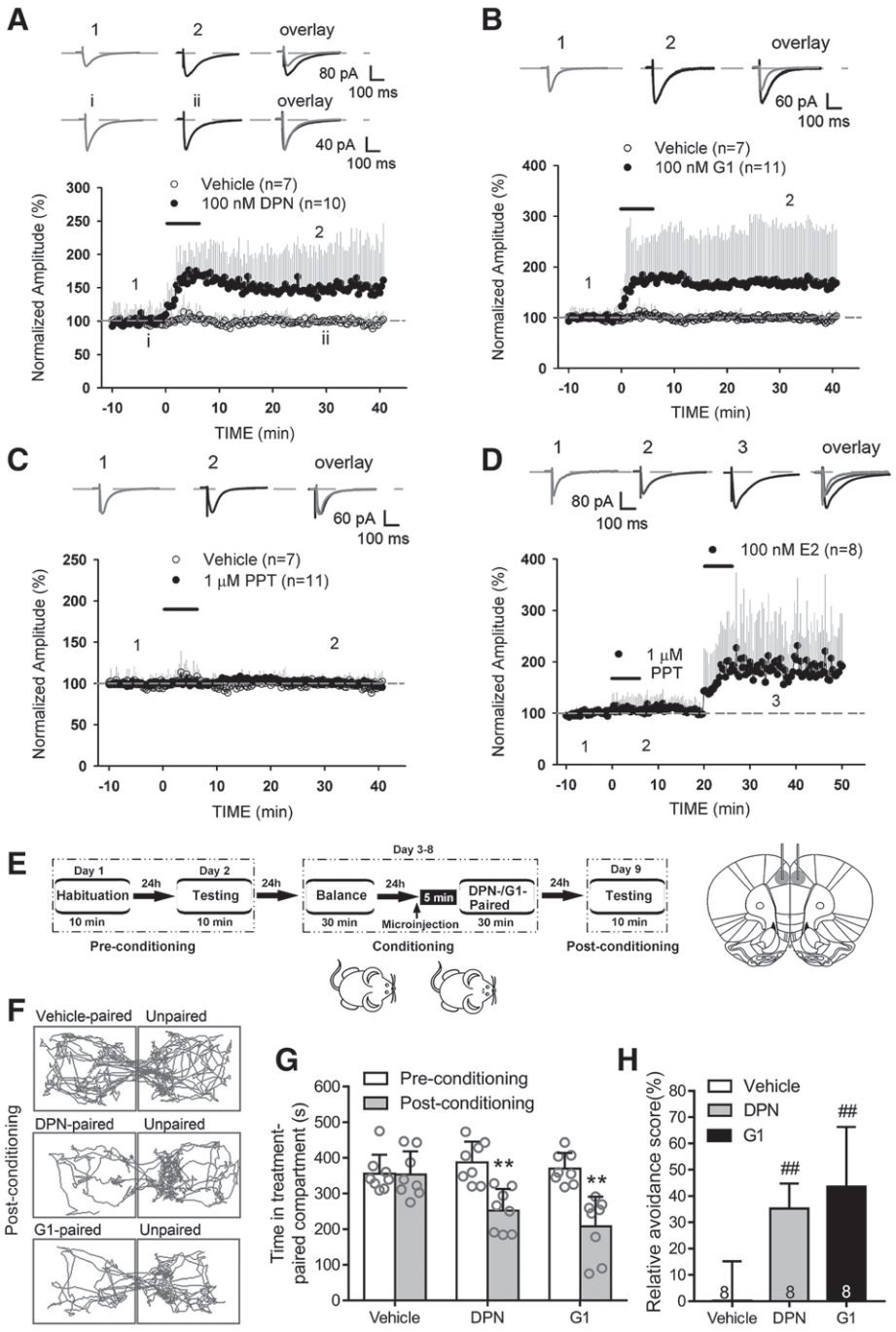

Fig. 5. *N*-Methyl-D-aspartate (NMDA)–mediated long-term potentiation and conditioned place avoidance are elicited by the activation of estrogen receptor-β and G protein–coupled estrogen receptor-1 but not estrogen receptor-α. (*A–C*) The estrogen receptor-β agonist 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN; *A*) and the G protein–coupled estrogen receptor-1 agonist (±)-1-[(3aR*,4S*,9bS*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta [c]quinolin-8-yl]-ethanone (G1; *B*) induces the long-term potentiation of NMDA-mediated excitatory postsynaptic currents (NMDA–long-term potentiation) in pyramidal neurons in rostral anterior cingulate cortex slices. n = 7 and 10. Vehicle (0.1% dimethyl sulfoxide) and the estrogen receptor-α agonist 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT; *C*) did not affect NMDA receptor–mediated excitatory postsynaptic currents; n = 7 and 11. (*D*) Exposure to 17β-estradiol (E2) after PPT application elicited NMDA-mediated long-term potentiation; n = 8. (*E*) Schematic of the protocol for experiments *F* to *H*. (*F–H*) Microinjections of the estrogen receptor-β agonist DPN (0.02 ng/μl, 0.5 μl/hemisphere) or the G protein–coupled estrogen receptor-1 agonist G1 (0.2 μg/μl, 0.5 μl/hemisphere) directly into the rostral anterior cingulate cortex–induced conditioned place avoidance. $^{**}P < 0.001$ *versus* the preconditioning test (two-tailed paired *t* test); $^{\#\#}P < 0.01$ *versus* vehicle (10% dimethyl sulfoxide; one-way ANOVA followed by *post hoc* Dunnett's test); n = 8, 8, and 8.

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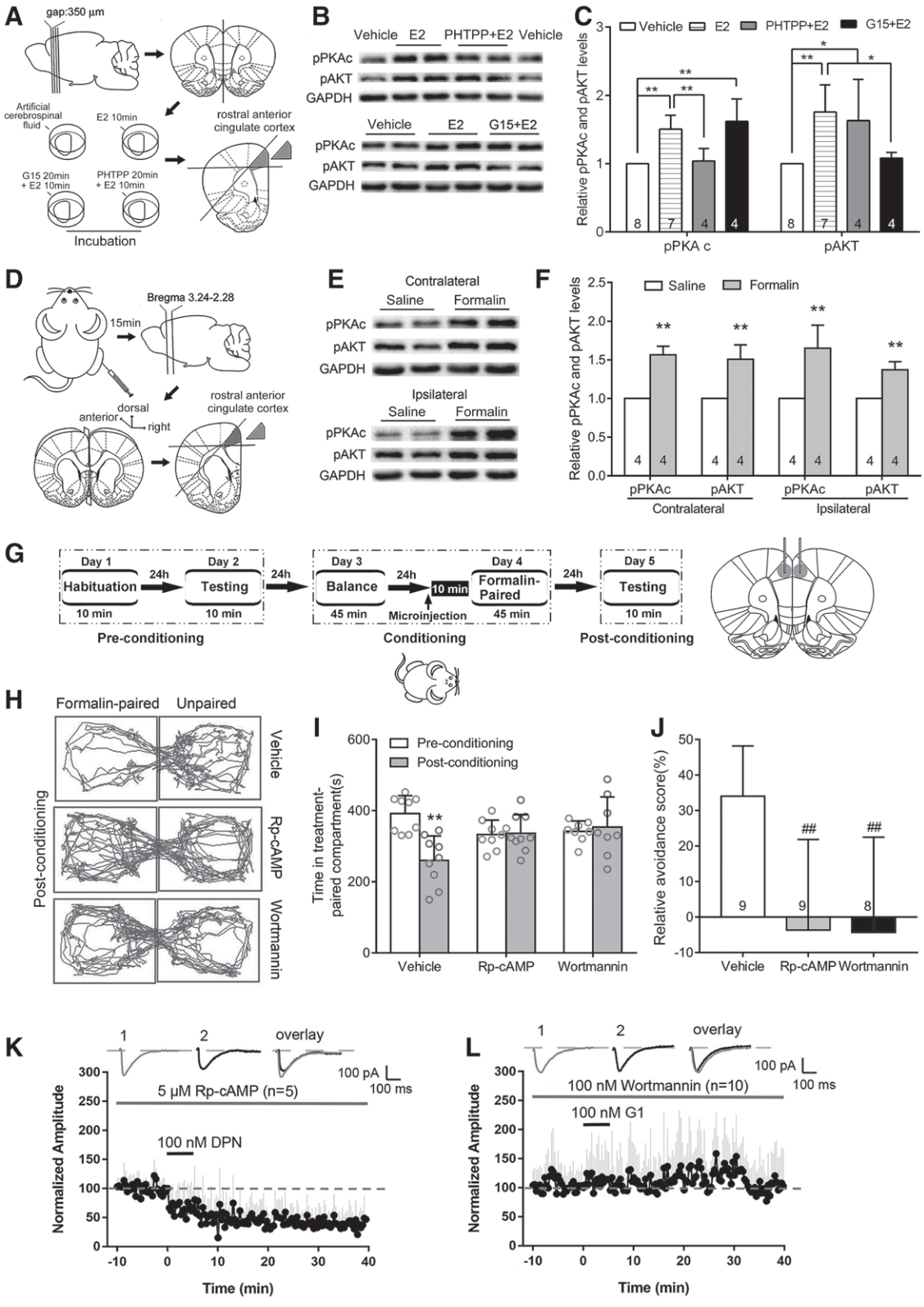

Fig. 6. Protein kinase A or protein kinase B signaling in the rostral anterior cingulate cortex mediates formalin-induced conditioned place avoidance acquisition and *N*-methyl-D-aspartate (NMDA)–mediated long-term potentiation. (*A*) Schematic of the protocol for the experiments in *B* and *C*. (*B*, *C*) Western blot analysis revealed increase in the phosphorylated protein kinase A catalytic subunit (pPKAc) and phosphorylated protein kinase B (pAKT)

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relative avoidance scores were much lower in the Rp-cAMP– and wortmannin-treated groups than in the vehicle group (mean ± SD: Rp-cAMP: −3.7 ± 14.1% and wortmannin: −4.3 ± 26.8% *vs.* vehicle: 34.0 ± 14.1%; one-way ANOVA, $F_{(2,23)} = 10.75$, $P = 0.001$; fig. 6J). These results suggest that the protein kinase A and protein kinase B signaling pathways may mediate the effect of estrogen receptor-β and G protein–coupled estrogen receptor-1, respectively, on pain-related aversion. To determine whether the regulation of NMDA-mediated long-term potentiation by estrogen receptor-β or G protein–coupled estrogen receptor-1 is dependent on cAMP/protein kinase A or phosphoinositide 3-kinase/protein kinase B signaling, we preincubated slices with the protein kinase A inhibitor Rp-cAMP (5 μM) or the protein kinase B inhibitor wortmannin (100 nM). The results showed that DPN–induced NMDA-mediated long-term potentiation was reversed by Rp-cAMP (fig. 6K), whereas G1–induced NMDA-mediated long-term potentiation was completely prevented by wortmannin (fig. 6L). Together with the results from our aforementioned formalin-induced conditioned place avoidance experiments, these results suggest that the estrogen receptor-β/protein kinase A and G protein–coupled estrogen receptor-1/protein kinase B pathways mediate pain-related negative emotion by rapidly modulating NMDA receptor–dependent excitatory synaptic transmission in rostral anterior cingulate cortex pyramidal neurons.

Fig. 6. (*Continued*). after exposure to 17β-estradiol (E2; 1 μM) for 10 min. Preincubation with the estrogen receptor-β antagonist 4-(2-phenyl-5,7-bis [trifluoromethyl] pyrazolo [1,5-a] pyrimidin-3-yl) phenol (PHTPP; 1 μM) significantly reduced E2–induced phosphorylated protein kinase A catalytic subunit (pPKAc) protein levels but not phosphorylated protein kinase B levels (pAKT). In contrast, the G protein–coupled estrogen receptor-1 antagonist (3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta [c] quinolone (G15;1 μM) blocked E2–induced pAKT but not pPKAc. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control $^{*}P < 0.05$; $^{**}P < 0.01$ (one-way ANOVA followed by *post hoc* Dunnett's test); n = 8, 7, 4, and 4. (*D*) Schematic of the protocol for the experiments in *E* and *F*. (*E*, *F*) Western blot for pPKAc and pAKT in the ipsilateral and contralateral rostral anterior cingulate cortex 15 min after intraplantar injection of formalin injection in rats. Both pPKAc and pAKT were increased in the bilateral rostral anterior cingulate cortex after formalin injection. $^{**}P < 0.01$ *versus* normal saline (two-tailed Student's *t* test); n = 4 and 4. (*G*) Schematic of the protocol for the experiments in *H* to *J*. (*H–J*) Microinjections of the protein kinase A inhibitor Rp-cyclic adenosine monophosphate (Rp-cAMP; 1 μg/μl, 0.5 μl/hemisphere) or the protein kinase B inhibitor wortmannin (0.2 μg/μl, 0.5 μl/hemisphere) into the rostral anterior cingulate cortex blocked formalin-induced conditioned place avoidance induction. $^{**}P < 0.01$ *versus* the preconditioning test (two tailed paired *t* test); $^{\#\#}P < 0.01$ *versus* vehicle (one-way ANOVA followed by *post hoc* Dunnett's test); n = 9, 9, and 8. (*K*, *L*) Bath application of the protein kinase A inhibitor Rp-cAMP (*K*) or the protein kinase B inhibitor wortmannin (*L*) markedly blocked 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN)- and (±)-1-[(3aR*,4S*,9bS*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta [c]quinolin-8-yl]-ethanone–(G1)-induced NMDA-mediated long-term potentiation, respectively; n = 5 and 10. The *insets* show the averages of two to three excitatory postsynaptic currents before and after DPN/G1/E2 or Rp-cAMP/wortmannin perfusion. The *dashed line* indicates the mean basal synaptic responses.

Discussion

Previous studies have shown that excitatory amino acids and their receptors, especially NMDA receptors, are critically involved in pain-related aversion.[22,28,29] Moreover, we revealed that brain-derived estrogen, as a neuromodulator, in the rostral anterior cingulate cortex drives the emotional component of pain through the modulation of the NMDA receptors.[4] However, the roles of different estrogen receptors and downstream signaling pathways are still unknown. Here, we provide preclinical evidence regarding the negative emotion of chronic pain using a formalin-induced conditioned place avoidance and place escape/avoidance paradigm to evaluate pain-related aversion to investigate the roles of estrogen receptors in pain-related aversion. We found that most excitatory pyramidal neurons in the rostral anterior cingulate cortex expressed estrogen receptors, and different estrogen receptors have divergent functions and separate downstream signaling pathways to contribute to pain-related aversion. The blockade of estrogen receptor-β and G protein–coupled estrogen receptor-1 but not estrogen receptor-α eliminated formalin-induced conditioned place avoidance and place escape/avoidance. The activation of estrogen receptor-β and G protein–coupled estrogen receptor-1 evoked NMDA receptor–dependent long-term potentiation in the rostral anterior cingulate cortex and directly elicited conditioned place avoidance, which was specifically mediated by the estrogen receptor-β/protein kinase A and G protein–coupled estrogen receptor-1/protein kinase B pathways. These results suggest that estrogen receptor-β/cAMP/protein kinase A and G protein–coupled estrogen receptor-1/phosphoinositide 3-kinase/protein kinase B signaling rapidly regulates NMDA receptor–dependent synaptic plasticity in rostral anterior cingulate cortex neurons to participate in pain-related aversion. A novel mechanism-based therapeutic strategy for pain-related emotional disorders might be proposed.

Activation of Estrogen Receptor-β and G Protein–coupled Estrogen Receptor-1 in the Rostral Anterior Cingulate Cortex Plays a Critical Role in Pain-related Aversion

The intraplantar injection of diluted formalin induces conditioned place avoidance and other pain-like behaviors in rodents, indicating that formalin induces aversive responses in rodents that resemble responses to noxious stimuli in humans. In the formalin-induced conditioned place avoidance paradigm, the presence of nociception is associated with the conditioning environment.[34] Using formalin-induced conditioned place

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avoidance, Johansen *et al.*[27] demonstrated that the destruction of rostral anterior cingulate cortex neurons blocks formalin-induced aversion but does not affect the induction of other pain-like behaviors (such as licking, flinching, and elevating) by formalin, providing direct evidence for the separation of the sensory–discriminative and affective–motivational components of pain. Our previous study found that intraplantar injection of formalin results in conditioned place avoidance and 17β-estradiol release in the rostral anterior cingulate cortex. The inhibition of endogenous 17β-estradiol synthesis by androstatrienedione, an inhibitor of aromatase (a key enzyme required for estrogen production), prevents formalin-induced conditioned place avoidance and attenuates extracellular 17β-estradiol levels in the rostral anterior cingulate cortex without sexual differences.[4] This lack of sex specificity in formalin-induced 17β-estradiol release and conditioned place avoidance suggests that brain-derived estrogen is critical for pain-related aversion in both sexes. In the present study, we further revealed that the blockade of estrogen receptor-β or G protein–coupled estrogen receptor-1 in the rostral anterior cingulate cortex eliminated formalin-induced conditioned place avoidance and place escape/avoidance, providing new support for endogenous 17β-estradiol–mediated pain-related aversion. The system injection of 17β-estradiol or the injection of 17β-estradiol into the rostral anterior cingulate cortex has been reported to produce aversive behaviors in both male and female animals in conditioned taste aversion and conditioned place avoidance tests.[35,36] The current study showed that of the direction administration of DPN or G1 into the rostral anterior cingulate cortex elicited conditioned place avoidance but did not affect basal thermal or mechanical sensitivity, implying that estrogen receptor-β and G protein–coupled estrogen receptor-1 play important roles in estrogen-induced aversive learning. Intriguingly, although all three estrogen receptors are abundantly expressed in the rostral anterior cingulate cortex, estrogen receptor-α does not seem to be required for the induction of pain-related aversion. It has been reported that the three estrogen receptors appear to play different roles depending on the task and brain region. For example, estrogen receptor-α and G protein–coupled estrogen receptor-1 agonists rapidly facilitate short-term social and object recognition and spatial memory, whereas an estrogen receptor-β agonist only rapidly improves short-term spatial memory when administered systemically or into the hippocampus.[37,38] In the medial amygdala, all three estrogen receptors facilitate social recognition.[37–39] Thus, specifically targeting the exact estrogen receptor involved in different situations might be a more efficient strategy for the treatment of distinct estrogen-related disorders that induces fewer side effects.

Activation of Estrogen Receptor-β and G Protein–coupled Estrogen Receptor-1 Facilitates NMDA Receptor–dependent Excitatory Synaptic Transmission and Plasticity in Rostral Anterior Cingulate Cortex Neurons

The present study showed that all three estrogen receptors were mostly colocalized with the neuronal marker βIII-tubulin and were expressed in almost CaMK2α-positive excitatory pyramidal neurons in the rostral anterior cingulate cortex. Consistent with previous studies,[9,40] estrogen receptor-α and estrogen receptor-β were present in the cell nucleus, membrane, and cytoplasm, whereas G protein–coupled estrogen receptor-1 was only localized in the membrane and cytoplasm. More specifically, estrogen receptors are expressed not only on the plasma membrane of soma but also along dendritic shafts and in the synaptic and extrasynaptic regions within the dendritic spines of pyramidal neurons.[41–43] Consistently, we also detected the colocalization of estrogen receptor-α and estrogen receptor-β with the neuronal dendrite marker MAP-2 and all three estrogen receptors in synaptosomal membrane proteins from rostral anterior cingulate cortex tissues, implying that estrogen receptors have synaptic functions. 17β-Estradiol or the estrogen receptor agonist WAY 200070 causes a rapid increase in the synaptic response in hippocampal and rostral anterior cingulate cortex slices.[44] In hippocampal slices, the estrogen receptor-β selective agonist DPN but not the estrogen receptor-α agonist PPT mimics the effects of estrogen on the regulation of synaptic transmission and plasticity.[17,44] Liu *et al.*[15] observed that estrogen receptor-β activation significantly enhances θ burst stimulation-induced long-term potentiation in wild-type mice but not in $Esr2^{-/-}$ mice. Our previous study in spinal cord slices showed that DPN but not PPT persistently increases NMDA receptor–dependent synaptic transmission and dendritic spine density.[45] In addition to classical estrogen receptors, the nonclassical G protein–coupled estrogen receptor-1 is also involved in the estrogen-induced synaptic response. The G protein–coupled estrogen receptor-1 agonists G1 and STX, a synthetic diphenylacrylamide compound, robustly potentiate hippocampal excitatory synaptic transmission and synaptic plasticity.[46,47] Interestingly, Oberlander and Woolley[48] found that in hippocampal slices from rats, an estrogen receptor-β agonist potentiates glutamatergic synapses postsynaptically in males, whereas in females, this effect is induced by G1. Presynaptically, 17β-estradiol–induced synaptic potentiation is mimicked by estrogen receptor-α agonist in males and estrogen receptor-β agonist in females, respectively. These results indicate that the functions of estrogen receptors at synapses are likely to be heterogeneous. Correspondingly, our current study further demonstrated that the activation of estrogen receptor-β or G protein–coupled estrogen receptor-1 but not estrogen receptor-α mimicked the sustained effects of 17β-estradiol on NMDA-mediated excitatory postsynaptic currents in rostral anterior cingulate cortex neurons. Providing further support, we also observed that 17β-estradiol treatment rapidly upregulated synaptosomal membrane estrogen receptor-β and G protein–coupled estrogen receptor-1 levels but did not affect estrogen receptor-α levels. DPN and G1 but not PPT robustly augmented the expression of the NMDA receptor subunits GluN1 and GluN2B in the synaptosomal

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membrane. These data further confirmed our behavioral findings that estrogen receptor-β and G protein–coupled estrogen receptor-1 but not estrogen receptor-α were required in the induction of pain-related aversion. Extranuclear estrogen receptors couple to different second messenger signaling cascades to initiate rapid nongenomic mechanisms.[49,50] In the present study, we determined that estrogen receptor-β activated the cAMP/protein kinase A pathway and that G protein–coupled estrogen receptor-1 triggered phosphoinositide 3-kinase/protein kinase B signaling, rapidly modulating NMDA receptor–dependent synaptic transmission and plasticity in rostral anterior cingulate cortex neurons to drive pain-related aversion (fig. 7).

This study has several limitations. For example, although estrogen receptors were mainly expressed in CaMK2α-positive excitatory pyramidal neurons in the rostral anterior cingulate cortex, estrogen receptors were detected in CaMK2α-negative neurons (fig. 1), suggesting that estrogen receptors were also expressed on inhibitory interneurons. Further study is needed to determine whether estrogen receptors expressed by CaMK2α-negative neurons are involved in pain-related aversion. In addition, we have demonstrated that brain-derived estrogen drives pain-related negative emotion without sex specificity[4] and that the mRNAs of the three estrogen receptors are expressed in the rostral anterior cingulate cortex without sex differences. Thus, male animals were primarily used in the current study to exclude the influence of the estrous cycle of females. However, we cannot exclude the distinct roles of the different estrogen receptors in female and male animals. Moreover, estrogen receptor-α does not seem to be required for the induction of pain-related aversion, although it is abundantly expressed in the rostral anterior cingulate cortex. To date, we do not know its role in the rostral anterior cingulate cortex. Finally, we also suggest using an automatic recording system to monitor formalin-induced nociceptive responses during formalin-paired training in the formalin-induced conditioned place avoidance paradigm,[22] which will facilitate our research and minimize the number of experimental animals.

In conclusion, this study identified the distinct roles of the three estrogen receptors in pain-related aversion and filled some gaps in knowledge regarding the interactions between estrogen and the receptors of excitatory amino acids, further elucidating the mechanisms by which brain-derived estrogen contributes to pain-related aversion.

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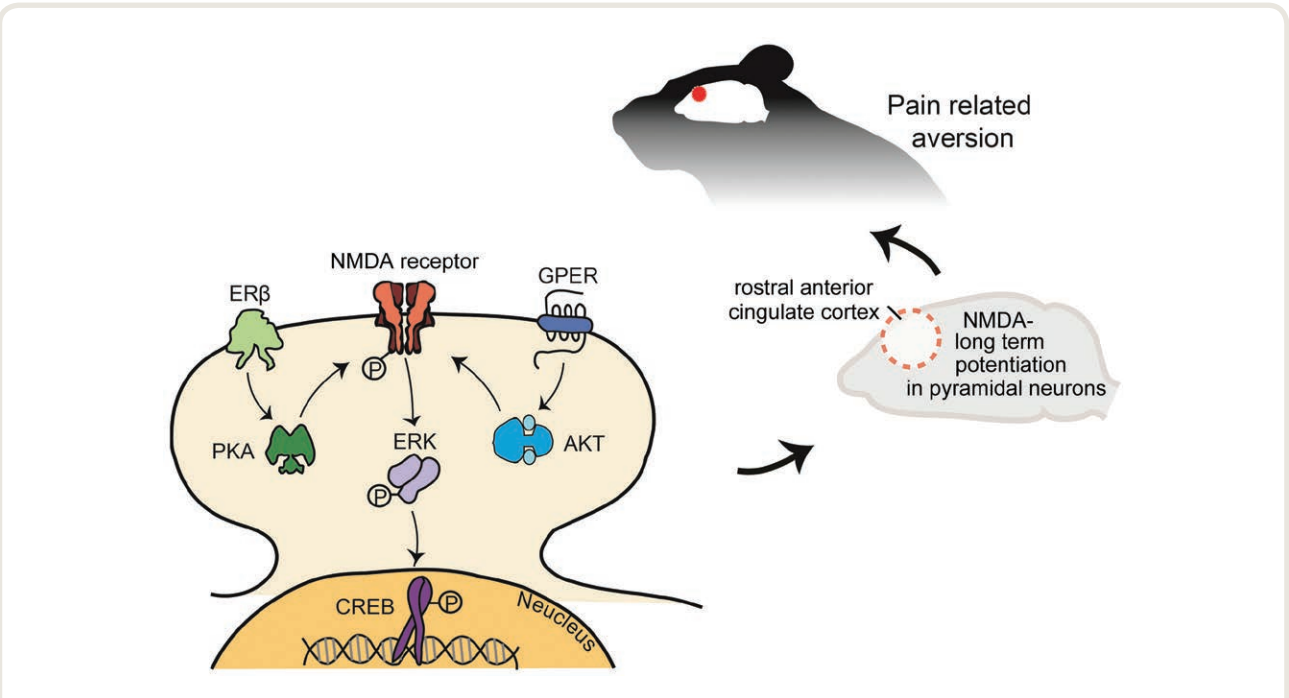

Fig. 7. Putative model of the underlying mechanisms by which estrogen receptors in the rostral anterior cingulate cortex mediate the emotional component of pain. Estrogen in the anterior cingulate cortex drives the emotional component of pain *via* the estrogen receptor-β (ERβ)/protein kinase A (PKA) and G protein–coupled estrogen receptor-1 (GPER)/protein kinase B (AKT) pathways to facilitate NMDA receptor–mediated synaptic transmission and plasticity (see Discussion for details). CREB, cAMP-response element binding protein; ERK, extracellular regulated protein kinases.

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Competing Interests

The authors declare no competing interests.

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After winning Pennsylvania's intercollegiate St. Andrews Society scholarship competition, I studied natural philosophy at Scotland's University of Edinburgh (1975 to 1976). My physics lab instructor was Edinburgh's venerable James Kyles, FRSE, who had collaborated with neutron discoverer James Chadwick. As the visiting Andrew Mutch Scholar to the Scottish Universities Research Reactor Centre (photographed by me, *left*), I recorded Dr. Keith Boddy's use of particle physics to investigate cadmium poisoning. Decades after these *particular* inspirations, I would revisit the Scottish capital for *general* inspiration or, more accurately, general inhalation. In 2004 and then 2014, as a medical antiques courier, I would hand-carry rare acquisitions, c. 1846 Hooper and Squire Ether Inhalers (*right upper* and *lower*, respectively) from an antiques dealer in Edinburgh back to Chicagoland's Wood Library-Museum. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

George S. Bause, M.D., M.P.H., Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio.

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