Estrogen in the Anterior Cingulate Cortex Contributes to Pain-Related Aversion

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The rostral anterior cingulate cortex (rACC) is a key structure of pain affect. Whether and how estrogen in the rACC regulates pain-related negative emotion remains unclear. Behaviorally, using formalin-induced conditioned place aversion (F-CPA) in rats, which is believed to reflect the pain-related negative emotion, we found that estrogen receptor (ER) inhibitor ICI 182, 780 (ICI, 7α,17β)–[9-{(4,4,5,5,5-Pentafluoropentyl)sulfanyl}mononyl]estr-1,3,5(10)-triene-3,17-diol or inhibitor of aromatase androstatrienedione into the rACC completely blocked F-CPA in either sex. An analogous effect was also observed in ovariectomized rats. Furthermore, exogenous estrogen in the absence of a formalin noxious stimulus was sufficient to elicit CPA (E-CPA) in both sexes by activating the membrane estrogen receptors (mERs) and N-methyl-D-aspartic acid (NMDA) receptors (NMDARs). Electrophysiologically, we demonstrated that estrogen acutely enhanced the glutamatergic excitatory postsynaptic currents (EPSCs) in rACC slices by increasing the ratio of NMDA–EPSCs to α-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propionic acid–EPSCs and presynaptic glutamate release. Interestingly, a brief exposure to estrogen elicited a persistent enhancement of NMDA–EPSCs, and this NMDA–long-term potentiation required the activation of the mERs, protein kinase A and NMDAR subunit NR2B. Finally, estrogen induced rapid dendritic spine formation in cultured rACC neurons. These results suggest that estrogen in the rACC, as a neuromodulator, drives affective pain via facilitating NMDA receptor-mediated synaptic transmission.

Keywords: anterior cingulate cortex, estrogen and estrogen receptors, formalin-induced conditioned place aversion, long-term potentiation, pain-related aversion

Introduction

Pain is a complex experience that incorporates both sensory and affective dimensions. The sensory aspect describes the location, intensity, and quality of somatosensory stimuli. The affective aspect attributes emotional coloring to the pain experience, producing the desire to terminate or escape from the noxious stimuli (Zhang 2006). Clinical observations have increasingly indicated that in chronic pain patients, pain-related negative affect is more disabling than the pain itself (Crombez et al. 1999). Thus, research on pain-related negative affect has received increasing research attention.

The anterior cingulate cortex (ACC) is implicated in many functions related to emotional processing (Etkin et al. 2011). Previous studies have demonstrated that the destruction of neurons originating from the rostral ACC (rACC) blocks formalin-induced conditioned place aversion (F-CPA), a pain-related aversive learning that directly reflects the affective component of pain in rats, without reducing acute pain-related behaviors (Johansen et al. 2001; Gao et al. 2004).

Additionally, the direct activation of rACC neurons by the microinjection of excitatory amino acid can produce CPA in the absence of a peripheral noxious stimulus, suggesting that neurons in the rACC mediate both pain-induced negative affect and a nociceptor-driven aversive teaching signal (Johansen and Fields 2004). Moreover, studies from our laboratory revealed that the activation of N-methyl-D-aspartic acid (NMDA) receptors (NMDARs) and the protein kinase A (PKA)–extracellular signal-related kinase (ERK)–cyclic adenosine monophosphate response element-binding protein (CREB) signaling pathway in the rACC is required for the induction of pain-related negative emotion (Ren et al. 2006; Cao, Gao, et al. 2009; Li et al. 2009). Actually, this signaling pathway is also strongly implicated in long-lasting neuronal plasticity (Impney et al. 1998; Toyoda et al. 2007). However, it remains unclear whether the nociceptor-driven neuronal plasticity is fundamental to pain-related aversive emotion.

Estrogen, a steroid hormone, is involved across an extensive spectrum of neural functions, including learning, memory, nociception, and mood disorder (Ji, Murphy, et al. 2003; Evrard and Balthazart 2004; Hojo et al. 2008; Shansky et al. 2010). 17β-estradiol (E2), a main form of estrogen, acts by binding and activating both intracellular estrogen receptors (ERs) and receptors associated with the plasma membrane (mERs). E2 has been reported to enhance NMDA-mediated synaptic activity and to promote the formation of new dendritic spines and excitatory synapses, not only slowly but also rapidly, in the hippocampus and cortex (Smith and McMahon 2005, 2006; Fatehi et al. 2006; Feltehi et al. 2006; Feltehi et al. 2007; Schwarz et al. 2008; Srivastava et al. 2008). Besides the circulating gonadal hormones into the brain, estrogen is locally synthesized from androgen precursors, such as testosterone, or directly from the cholesterol de novo in the brain, in areas such as the hippocampus and cortex (Balthazart and Ball 2006; Hojo et al. 2006). For brain-derived estrogen with a higher concentration than the circulating hormone, the rapid modulation of synaptic plasticity and cognitive functions may be their essential functions (Balthazart and Ball 2006; Srivastava et al. 2008; Kramar et al. 2009). Given the similarities in molecular mechanisms underlying synaptic plasticity in different regions of the central nervous system (Ji, Kohno, et al. 2003), we hypothesized that affective pain in the rACC and learning memory in the hippocampus as well as persistent pain-induced central sensitization in the spinal cord may share common signaling pathways. In this study, we investigated whether and how estrogen in the rACC regulates affective pain and synaptic plasticity. We demonstrated that E2 rapidly facilitated NMDA-mediated excitatory synaptic transmission and plasticity in rACC neurons, which may drive the formation of pain-related negative emotion.
Methods

Animals and Reagents

Behavioral and electrophysiological experiments were performed on adult (2–3 month old) and young (2–3 week old, without estrous cycle) Sprague Dawley rats of both sexes, respectively. Rats were obtained from the Experimental Animal Center of the Chinese Academy of Science and were on a 12:12 h light/dark cycle with food and water available ad libitum. All experiments were performed in accordance with the guidelines of the International Association for the Study of Pain, and were approved by the Shanghai Animal Care and Use Committee.

All reagents were purchased from Sigma unless otherwise noted. The steroid 17β-estradiol (E2, a main form of estrogen) and ICI 182, 870, 870 (ICI, 7α,17β-[9-(4,4,5,5-Pentfluoropentyl)sulfonyl]nonylestra-1,3,5(10)-triene-3,17-diol) were dissolved in sesame oil or ethanol. E2-BSA, m-2-amino-5-phosphonovaleric acid (APV), and Homocysteic acid (HCA) were dissolved in normal saline (NS). Androstrenedione (ATD, Steroids) was prepared in ethyl acetate. Stock solutions of the following drugs were prepared in double-distilled water (ddH2O): bicuculline methiodide (BMI), APV, NMDA, α-aminoo-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA), H89, and Ifenprodil. guanosine diphosphate (GDP)-β-s was dissolved in intracellular solution.

Surgery and Drug Infusions

For removal of ovarian hormones, adult female rats were bilaterally ovarioctomized (OVX) under isoflurane anesthesia. Sham surgery included all aspects of the OVX procedure, except the fallopian tube and ovaries were left intact. After 2-week recovery from OVX surgery, rats were implanted with stainless steel cannula for intra-rACC drug infusions.

For microinjection studies, rats were anesthetized with intraperitoneal chloral hydrate (40 mg/kg), and secured placed into a stereotaxic device with bregma and lambda horizontally level. A 30-gauge stainless steel cannula with a 33-gauge stainless steel stylet plug was bilaterally implanted 0.5 mm above the rACC injection site [anteroposterior (AP) +2.6, mediolateral (ML) ± 0.6, dorsoventral (DV) –2.0] and the prefrontal cortex (PFC) [AP +2.6, ML ± 0.6, DV –3.7] according to the atlas of Paxinos and Watson (1998). Animals were allowed to recover for one week before the next experimental procedure. At the end of the experiment, brains were sectioned for cresyl violet staining to verify cannula position and injection site.

Rats were briefly anaesthetized with isoflurane, and microinjection was performed through a 33-gauge stainless-steel injection cannula that extended 0.5 mm beyond the tip of the guide cannula. The injection cannula was connected to a 1-μL Hamilton syringe with PE-10 tubing. A volume of 0.6 μL per hemisphere of either vehicle or drug was injected over a 5-min period. The injection cannula was left in place for an additional 5 min to minimize spread of the drug along the injection track.

Conditioned Place Aversion

The place conditioning apparatus and CPA procedure were as described previously with slight modifications (Cao, Gao, et al. 2009). The place conditioning apparatus consists of 3 opaque acrylic compartments (1 neutral chamber and 2 conditioning chambers with distinct olfactory, visual, and tactile cues). The experimental process consists of 3 distinct sessions: a preconditioning session, a conditioning session and a test (postconditioning) session. F-CPA task processing requires 3 days. Day 1 is the preconditioning day. At the beginning, a rat was placed in the neutral compartment. After habituating for 2 min, the entrance to each conditioning compartment was opened. When the rat enters any conditioning compartment, the doors connecting neutral and conditioning compartments were closed. The rat was allowed to explore the 2 conditioning compartments freely for 15 min. A timer automatically recorded the time spent in each of the compartments in a blind manner. Rats that spent >80% (720 s) on one side on that day were eliminated from the subsequent experiments. Day 2 is conditioning day. On this day, all doors were closed. The rat received no treatment in the morning and was randomly confined to one of the conditioning compartments for 45 min. After at least 6 h, in the afternoon, F-CPA training rat was given a unilateral hindpaw intraplantar (i.pl.) injection of 50 μL 5% formalin (or NS as control) and then restrained in the other conditioning compartment for 45 min. Day 3 is postconditioning day. The procedure was the same as day 1. The time animals spent in each compartment was measured. For estrogen and HCA experiments, the CPA procedure consisted of 5 days. Pre- (day 1) and post-tests (day 5) were the same as for F-CPA. Days 2–4 were conditioning days. On day 2, in the morning, the rat received no treatment and was randomly confined to one of the conditioning compartments for 45 min. After at least 6 h, in the afternoon, the rat received a bilateral intra-rACC microinjection of E2 (20 μM, 0.6 μL per side), and then restrained in the other conditioning compartment for 45 min. The same trials as day 2 were repeated on day 3 and day 4. In different conditioning groups, the compartments were counterbalanced.

Microdialysis and Estradiol Assay in the rACC and PFC

The samples from slice perfusate and microdialysis were collected. The microdialysis probe (MD2200, BAS, United States of America) was inserted into the unilateral ACC or PFC via the guide cannula to 1 mm beyond the tip of the guide cannula. The dialysis probe was connected to a microinfusing pump (BAS, Microdialysis Syringe 1.0 mL). The probe was perfused with artificial cerebrospinal fluid (ACSF) at a flow rate of 2.0 μL/min. After dialysate levels stabilized (about 1 h), a 40-min sample was collected as baseline values, and then formalin was injected in ipsilateral i. pl to the dialyzed rACC and PFC. In some animals, ATD (200 μM, 0.6 μL) was microinjected into the rACC 5 min before formalin and a 40-min sample was collected after formalin injection. Aliquots (200 μL) were frozen at –80°C for later analysis. The concentrations of E2 from dialysate and slice perfusate were determined by double-antibody radioimmunoassay kits according to protocols provided by the manufacturer (National Atomic Energy Research Institute, Beijing, China). The sensitivity of the kit was 1.4 pg/mL.

Formalin Test

Formalin (5% 50 μL) was injected into i. pl of the unilateral hindpaw. The lifting and licking time of the affected paw during each 5 min interval for 45 min after injection was recorded. A weighted pain score for each animal was calculated using the following formula (Tanimoto et al. 2003): formalin pain score = [(the time the animal spent on elevating injected paw + 2 × (the time the animal spent on licking or biting injected paw))/300].

Slice Preparation

Coronal brain slices containing the rACC were obtained from young rats (15–21 days old) of both sexes. After anesthetizing with isoflurane, rats were decapitated. The brain was quickly removed and submerged in pre-oxygenated (95% O2, 5% CO2) cold ACSF at 4°C, containing 126 mM NaCl, 4.0 mM KCl, 1.25 mM MgCl2, 26 mM NaHCO3, 1.25 mM Na2HPO4, 2.5 mM CaCl2, and 10 mM glucose. The osmolality was adjusted to 290–320 mOsmol/L and the pH to 7.35. A tissue block containing the rACC was glued to the stage using cyanoacrylate glue. Slices (400 μm) were cut with a vibratome (Leica VT 1000S, Leica, Germany) and transferred to an oxygenated chamber at room temperature (RT, 24 ± 1°C) for at least 1 h before further processing.

For immunohistochemical experiments, the rACC slices were treated with different agonist (or activators) at RT: E2 (100 nM) was added to ACSF 10 min. NMDA receptor antagonist APV (50 μM) was added to ACSF for 40 min before and during stimulation of E2. Vehicle (0.1% ethanol) treated slices served as controls. Subsequently, the slices were rapidly immersed in cold 4% paraformaldehyde in PB, and fixed for 60 min. The slices were then washed in PBS and processed for pERK and pCREB immunostaining.

For electrophysiological recording, a single slice was then transferred to a recording chamber and continuously perfused with recording solution at a rate of 5 mL/min at RT.
**Whole-Cell Recordings**

Whole-cell recordings were obtained from pyramidal-shaped neurons in layer II/III of the rACC with a patch clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA, United States of America), under visual control using differential interference contrast and infrared optics via a water-immersion objective (DIC-IR; Leica DMFLSA, Germany). Evoked excitatory postsynaptic currents (EPSCs) were induced by repetitive stimulations that were delivered by a bipolar tungsten stimulating electrode placed in layer V of the rACC, at 0.05 Hz. Recording electrodes made from 1.5-mm glass capillaries were pulled on a Flaming-Brown micropipette puller (P-97, Shutter Instrument Co. Novato, CA, United States of America) and filled with an intracellular solution containing (in mM) 150 Gluconate, 8 NaCl, 0.4 ethylene glycol tetraacetic acid, 2 Mg-adenosine triphosphate, 0.3 Na-guanosine triphosphate, and 10 glutamic acid (2-hydroxyethyl)-1-piperazineethanesulfonic acid, with the pH adjusted to 7.2 and osmolarity adjusted to 300 mOsmol/L. QX-314 (5 mM) and GDP-β-S (1 mM) was added when necessary. With this solution, the recording electrodes exhibited a resistance of 2–6 MΩ. Signals were low-pass-filtered at 2 kHz and digitized at 5 kHz with a digitizer (Digidata 1322 and 1440, Axon Instruments, Foster City, CA, United States of America). Pyramidal-shaped neurons were easily identified by their shape and electrophysiological properties. The typical firing pattern of pyramidal neurons showed significant firing frequency adaptation whereas interneurons showed fast-spiking action potentials followed by pronounced hyperpolarization. The recordings were initially obtained in normal ACSF to identify the cell’s pattern. E2 (10 nM, 100 nM, and 1 μM) was applied in the perfuse. The NMDA-mediated component of EPSCs (NMDA-EPSCs) was recorded in ACSF containing 6-cyano-7-nitroquinoloxaline-2,3-dione (CNQX, 20 μM) and BMI (10 μM) and neurons were voltage clamped at −40 mV. To obtain the NMDA currents, the membrane potential was held at −40 mV, and the medium were switched to an ACSF solution with low Mg2+ (0.1 mM) and high Ca2+ (3.8 mM) to partially remove the Mg2+ block. The concentrations of drugs applied in the perfuse were the follows: 20 μM NDMA, 50 μM APV, 20 μM CNQX, and 1 μM to eratotoxin (TTX). TTX was included in the perfuse to polysynaptic phenomena (Martina et al. 2005). Theta-burst stimulation (TBS, 5 trains of burst with 4 pulses at 100 Hz, 200 ms intervals; repeat 4 times with intervals of 10 s) was used to induce long-term potentiation (LTP). In these experiments, BMI (10 μM) was always present to block gamma-aminobutyric acid A receptor mediated inhibitory synaptic currents. The series resistance (Rs) was monitored during recording. Excluded from the analysis were cells in which Rs deviated >20% or cells with Rs > 60 μΩ. Data were collected with pClamp9.2 software and analyzed using Clampfit 9.2.

**Immunohistochemistry/Immunocytochemistry**

For immunohistochemical experiments, the rACC slices were treated with E2 (10 nM) or vehicle (ethanol) for 10 min. NMDA receptors antagonist APV (50 μM) was added to ACSF 30 min before and during E2. Subsequently, the slices were rapidly immersed in cold 4% paraformaldehyde and fixed for 60 min, and then processed for immunostaining. Cultured rACC neurons grown on glass coverslips were stained without fixation by incubation with E2-BSA-FITC (1 μg/mL) in PBS for 30 min in dark at 37°C.

**Western Blots**

After defined survival times, rats were killed by an overdose of Chloral hydrate (80 mg/kg) and the brain was quickly removed. The rACC was dissected on ice using a Rat Brain Matrix (Stoelting Company). Briefly, 3 coronal brain slices (1 mm thick) containing rACC (AP 3.7–0.7 from bregma) were cut. Then the brain slices were separated into left and right halves from the sagittal suture. Finally, the rACC tissues were dissected using a surgical blade and rapidly frozen in liquid nitrogen. Frozen samples were homogenized in a lysis buffer (12.5 μL/mg tissue) containing a mixture of protease inhibitors (Roche) and Phenylmethanesulfonyl fluoride (PMSF, Sigma). After incubating in ice for 30 min, samples were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatants were used for western blotting. Isolation of cytoplasmic and/or membrane and nuclear protein extracts were achieved using the Compartment Protein Extraction Kit (Chemicon) and protocol. Protein concentrations were measured using a BCA Protein Assay kit (Pierce).

Equal amount of protein (~20 μg) was loaded and separated in 10% Tris–Tricine SDS-PAGE gel. The resolved proteins were transferred onto polyvinilidene difluoride membranes (Amersham Bioscience). The membranes were blocked in 10% non-fat milk for 2 h at RT, and incubated at 4°C overnight with rabbit anti-NR1 (1:4000, Sigma), rabbit anti-phospho-NR2B (1:1000, Millipore) and mouse anti-NR2B (1:2000, Neumob) primary antibody. The blots were then incubated with the secondary antibody goat anti-rabbit IgG conjugated with horseradish peroxidase (1:1000, Pierce), for 2 h at 4°C. The blots were probed with glyceraldehyde 3-phosphate dehydrogenase antibodies as a loading control. Signals were finally visualized using enhanced chemiluminescence (ECL, Pierce) and the bands were visualized with the ChemiDoc XRS system (Bio-Rad). All western blot analysis was performed at least thrice, and consistent results were obtained. Bio-Rad Image Analysis System was then used to measure the integrated optic density of the bands.

**Cell Culture and Transfections**

Primary cultures of rACC neurons were obtained from rat embryos at embryonic day 18. rACC was isolated and dissociated with trypsin, and cells were plated (1 × 103 to 1 × 105 cells/mL) in Neurobasal medium (GIBCO) supplemented with B27 (GIBCO), t-Glutamine (GIBCO) and 5% fetal bovine serum (FBS) (Sigma) on 60-mm dishes that were precoated with poly-β-lysine (0.1 mg/mL or 1 mg/mL, Sigma). Cells were fed twice weekly thereafter with Neurobasal medium prepared as above but without 5% FBS. rACC neurons were grown at 37°C in 5% CO2 on coverslips. Cultured neurons were transfected with green fluorescent protein (GFP)-lentivirus (Genechem, Shanghai) 3 days before imaging at 14 day in vitro.

**Live-Cell Imaging**

Three days after transfection, coverslips were imaged in a sealed chamber filled with Neurobasal medium with a Nikon (Tokyo, Japan) TE300 inverted microscope. Images were obtained using a Yokogawa spinning disk (Solamere Technology Group). Healthy neurons with overall pyramidal morphologies expressing GFP were identified and imaged every 5–8 min from 30 min before to 120 min after E2 treatment at 37°C in 5% CO2. At each time point, Z-stacks of images were collected and analyzed using Origin (Microcal Software).
Statistical Analysis

Data are presented as mean ± SEM. Student’s t-test, paired t-tests, 1-way, 2-way or 2-way RM ANOVA followed by post hoc Student–Newmann–Keuls test were used to identify significant differences. In all cases, P<0.05 was considered statistically significant.

Results

Endogenous Estrogen Contributes to the Acquisition of Pain-Related Aversion Without Sex Specificity

F-CPA, a learned behavior that directly reflects the affective component of pain in rats (Johansen et al. 2001), was employed in this study with slight modifications (Fig. 1A).

When a unilateral i.pl. injection of formalin (5%, 50 μL) was paired with a particular compartment in the place conditioning apparatus, both female and male rats spent less time in this compartment on the post-conditioning day compared with the pre-conditioning day (paired t-test, male: t0.01,5 = 5.316, P<0.01; female: t0.01,5 = 13.357, P<0.01). Control animals with an i.pl. injection of NS did not exhibit CPA (Fig. 1B). The CPA scores (the time spent in the treatment-paired compartment on the pre-conditioning day minus that on the post-conditioning day) were not significantly different between female and male groups in formalin-treated rats (male vs. female: 287.67 ± 54.11 vs. 273.00 ± 20.43, Student’s t-test, t0.05,10 = 0.254, P>0.05) (Fig. 1C).
Neurosteroid estrogen, as a neuromodulator, can be produced rapidly and locally in male and female brains, including the hippocampus and cortex (Hojo et al. 2004; Balthazart and Ball 2006; Yague et al. 2006). To investigate the contribution of endogenous estrogen in the induction of pain-related aversion, ERs antagonist ICI 182, 780 (ICI) was bilaterally microinjected into the rACC (20 μM, 0.6 μL per side) 5 min before formalin-paired conditioning (Fig. 1A). F-CPA acquisition was completely blocked by ICI in both female and male rats (Fig. 1B,C). Differently, when ICI was bilaterally microinjected into PFC instead of rACC, F-CPA acquisition could not be inhibited in either sex (Fig. 1D). Similarly, intra-rACC injection of androstatrienedione (ATD, 200 μM, 0.6 μL per side), an inhibitor of aromatase (a key enzyme required for estrogen production), 5 min before formalin-paired conditioning also prevented F-CPA acquisition in both sexes (2-way RM ANOVA treatment: \( F_{5,33} = 4.923, P < 0.01 \); time: \( F_{1,11} = 65.609, P < 0.01 \); sex: \( F_{1,13} = 1.398, P > 0.05 \) (Fig. 1B). In particular, we examined the effect of ATD on F-CPA in the rats which were subjected to bilateral OVX. F-CPA was reliably induced in OVX rats with significant difference in the time spent in the formalin-paired compartment between the pre- and post-conditioning day. When ATD was bilaterally microinjected into the rACC, OVX rats failed to produce CPA (Fig. 1E,F). Intriguingly, the F-CPA score in OVX group was significantly lower than that in sham OVX group (1-way ANOVA, \( F_{2,20} = 10.234, P < 0.01 \)), implying that ovarian E2 in adult female rats may also be involved in this process.

To confirm whether formalin stimulation rapidly results in local synthesis and release of estrogen, E2 concentration in extracellular dialysate of the rACC was measured. At a flow rate of 8 μL/min, the base dialysate concentration of E2 was 31.51 ± 6.53, 34.24 ± 4.67, and 36.87 ± 4.40 pmol/L in the rACC of male, female, and OVX rats, respectively (1-way ANOVA, \( F_{2,13} = 0.230, P > 0.05 \)). During 40 min after formalin injection, the dialysate E2 levels in the rACC were significantly increased compared with the basal dialysate concentration in all groups of male, female, and OVX rats. Intra-rACC ATD completely prevented formalin-induced increase in E2 concentration in the 3 groups, providing direct evidence for brain-derived estrogen in the rACC (2-way ANOVA followed by post-hoc Student–Newmann–Keuls test, treatment: \( F_{2,40} = 10.938, P < 0.01 \); sex: \( F_{2,40} = 0.0814, P > 0.05 \); treatment × sex: \( F_{4,80} = 0.947, P > 0.05 \) (Fig. 1G). In the PFC, there were similar base dialysate concentration of E2 in male, female, and OVX rats. However, no significant increases were observed in E2 level of the PFC dialysate following formalin injection (Fig. 1H).

Estrogen has been shown to play an important role in modulating pain sensitivity (Aloisi et al. 2002; Ji, Murphy, et al. 2003). To test whether the endogenous estrogen in the rACC is also important for formalin-induced spontaneous pain-like behavior, we examined the effects of an intra-rACC injection of ATD and ICI on formalin-induced biphasic nociceptive responses by manual detection. The first phase (i.e. early phase) began immediately after formalin injection and lasted for approximately 5 min. Following the first phase, there was one interphase, which lasted for about 10 min. During this phase, the rat was relatively quiet with rare licking or biting. The second phase (i.e. late phase) began about 15 min after formalin injection and lasted for about 30 min. As shown in Figure 1I, either ICI or ATD had no effect on formalin-induced nociceptive early phase (1-way ANOVA, \( F_{2,21} = 2.76, P > 0.05 \) ) and late phase responses (2-way ANOVA, treatment: \( F_{2,21} = 1.879, P > 0.05 \); treatment × time: \( F_{6,84} = 1.006, P > 0.05 \)), although a more depressed interphase was observed in ATD-treated groups (2-way ANOVA, \( F_{2,21} = 5.106, P < 0.05 \) (Fig. 1J). The locations of the ATD- and ICI-microinjected sites in the rACC and PFC are shown in Figure 1J.

Exogenous Estrogen Elicits CPA via NMDA Receptors

To test whether estrogen per se is sufficient to produce CPA, E2 (20 μM, 0.6 μL per side) was directly microinjected into the bilateral rACC without an i.pl. injection of formalin. Rats spent significantly less time in the E2-paired context on the postconditioning day compared with the preconditioning day (paired t-test, male: \( t_{9,01,10} = 4.070, P < 0.01 \); female: \( t_{0,01,10} = 6.569, P < 0.01 \) (Fig. 2A,B). Similar to F-CPA, The CPA scores of E2-induced CPA (E-CPA) is also not sex specific (male vs. female: 247.72 ± 60.86 vs. 229.36 ± 34.91). In the PFC, there were similar base dialysate concentration in the 3 groups, providing direct evidence for brain-derived estrogen in the rACC and PFC are shown in Figure 2D. Given that neither F-CPA nor E-CPA scores had significantly different between male and female groups, we therefore pooled sex group averages in the following experiments. As shown in Figure 2D, E-CPA acquisition can be blocked by pre-administration of ERs antagonist ICI (20 μM, 0.6 μL per side) into the rACC. Neither the intra-rACC vehicle (sesame oil, 0.6 μL per side) nor ICI alone produced CPA (Fig. 2). A membrane-impermeable estrogen conjugate E2-BSA (20 μM, 0.6 μL per side) that has been observed to act only on membrane ERs can mimic E2-induced CPA (Fig. 2D), which strongly indicates that E2-induced aversive learning is predominantly mediated by membrane ERs (mERs). Distributions of E2-BSA binding sites on the plasma membrane of cells were detected by E2-BSA-FITC (E2-BSA conjugated to FITC) in cultured rACC neurons (Fig. 2D).

Previous studies from ours and Fields’ laboratories have demonstrated that the activation of glutamate receptors, especially NMDAR in the rACC are necessary for the acquisition of F-CPA (Johanssen and Fields 2004; Lei et al. 2004; Li et al. 2009). Also, the crosstalk between E2 and NMDAR has been reported (Tang et al. 2008). We therefore microinjected selective NMDA receptor (NMDAR) antagonist APV (25 mM, 0.6 μL per side) into the rACC during E2 conditioning, and found that E-CPA was completely blocked, suggesting that E2-induced CPA may be mediated by NMDAR (Fig. 2F). As a support, we further demonstrated that NMDAR subunits NR1 and NR2B expression levels were significantly increased by E2 in the rACC following E-CPA conditioning (1-way ANOVA, \( F_{2,12} = 9.150, P < 0.01 \) (Fig. 2E). In addition, the colocalization of NMDAR subunit NR1 with ERα, ERβ, and GPR30 in cultured rACC neurons also provided a cellular basis for the involvement of NMDAR in the estrogenic effects (Fig. 2F).
et al. 2010). We next investigated the ability of E2 to rapidly regulate excitatory synaptic transmission in rACC neurons. Whole-cell recordings were performed in visually identified pyramidal neurons in layer II/III of rACC slices from 15- to 21-day-old rats of both sexes whose E2 concentrations had not detectable difference in perfusate of rACC slices. We identified pyramidal neurons based on the pyramidal shape of their somata and by injecting depolarized currents into neurons to induce action potentials (Mahanty and Sah 1998). The typical firing pattern of pyramidal neurons showed significant firing frequency adaptation whereas interneurons showed fast-spiking action potentials followed by pronounced hyperpolarization (Supplementary Fig. S1). Evoked EPSCs were obtained by delivering focal electrical stimulation to layer V of rACC, and neurons were voltage clamped at −40 mV. The addition of blockers of AMPA (CNQX, 20 μM) and NMDA (APV, 50 μM) receptors together completely abolished the EPSCs (data not shown). Bath application of E2 (10 nM, 100 nM, and 1 μM) rapidly potentiated EPSC amplitude within minutes in most of the recorded neurons (Fig. 3A). At 10 nM, E2 potentiated EPSCs in 7 of 20 neurons (35%); 100 nM E2 potentiated EPSCs in 12 of 17 neurons (71%); and 1 μM E2 potentiated EPSCs in 17 of 19 neurons (89%); these results indicate the increased frequency of EPSC potentiation with increasing concentrations of E2. This acute effect of E2 on EPSCs can be occluded in the presence of ICI (1 μM). A bath

**Figure 2.** Exogenous estrogen elicits CPA via estrogen receptors. (A) Schematic of the protocol for the experiments B–E. (B) Intra-rACC injections of E2 (20 μM, 0.6 μL per side) induce CPA in both sexes, as indicated by time spent in the treatment-paired compartment on preconditioning and postconditioning days. (C) Intra-PFC injections of E2 (20 μM, 0.6 μL per side) cannot induce CPA in both sexes. (D) Intra-rACC injections of E2 and membrane-impermeable estrogen conjugate E2-BSA (20 μM, 0.6 μL per side) but not vehicle, ICI182, 780 (ICI) or APV alone induce CPA. Pre-injection of ICI or NMDA receptor antagonist APV (25 mM, 0.6 μL per side) into the rACC attenuate CPA scores. Insert photograph for E2-BSA-FITC staining in cultured rACC neurons. Sex group averages were pooled in these experiments. (E) Western blot analysis reveals a significant increase of NR1 and NR2B in the rACC following E-CPA conditioning (rats received the same training trial on days 1–4 of the conditioning procedure as they received for E-CPA and were then sacrificed on day 5). Glyceraldehyde 3-phosphate dehydrogenase serves as loading control. (F) Double immunocytochemistry reveals the colocalization of ERα, ERβ and GPR30 with NMDA receptor subunit NR1 in cultured rACC neurons. **P < 0.01 versus preconditioning day; #P < 0.05, ##P < 0.01 versus vehicle; ^ ^P < 0.01 versus E2.
application of ICI alone had no effect on EPSCs (1-way ANOVA, \(F_{5,104} = 2.445, P < 0.05\)) (Fig. 3A).

Rapid regulation of E2 on excitatory synaptic transmission was further analysis by measuring the ratio of NMDAR- to AMPAR-mediated components of EPSCs. NMDA-EPSCs and AMPA-EPSCs were isolated electrophysiologically or pharmacologically (Fig. 3B and Supplementary Fig. S1B). To relate the changes in NMDA transmission to AMPA transmission, we calculated a ratio by dividing the average maximum NMDA-EPSCs by the average maximum AMPA-EPSCs (we refer to this ratio as the NMDA/AMPA ratio). E2 significantly increased the NMDA/AMPA ratio dose dependently, which could be occluded by ICI (1-way ANOVA, \(F_{4,100} = 10.066, P < 0.01\)) (Fig. 3B), indicating that estrogen modulated rACC neuron excitatory synaptic transmission under a distinct manner of NMDAR and AMPAR.

The effects of E2 on NMDA- and AMPA-evoked currents were further investigated. An application of NMDA (30 μM) for 60 s, by an addition of the compound to a low-Mg²⁺ (0.1 mM) and high-Ca²⁺ (3.8 mM) perfusion solution in the presence of TTX (1 μM) and CNQX (20 μM) at −40 mV, evoked an inward current. APV (50 μM) completely abolished the NMDA-currents. The average amplitude of the current in cells treated with 10 and 100 nM E2 was statistically greater than in the control (One-way ANOVA, \(F_{2,106} = 10.066, P < 0.01\)) (Fig. 3C), indicating that estrogen modulated rACC neuron excitatory synaptic transmission under a distinct manner of NMDAR and AMPAR.

To determine presynaptic effects of E2, we computed paired-pulse ratio (PPR), a measure related to neurotransmitter release probability that is commonly used to assess changes in presynaptic function (Zucker and Regehr 2002). Prior to E2 application, the second EPSC (EPSC₂), which was evoked by the second pair-pulse stimulation (interpulse interval: 60 ms), was compared with the first EPSC (EPSC₁), resulting in an EPSC₂/EPSC₁ ratio of 1.41 ± 0.04 (n = 23). E2 (10 nM or 100 nM) significantly decreased the PPR within minutes. ICI completely blocked E2-induced attenuation, as represented by the PPR (Supplementary Fig. S3A). This result suggests that the E2-induced potentiation of EPSC amplitude is mediated, at least in part, by increasing glutamate release probability. As a support, double immunofluorescence staining indicated that most glutamatergic neurons expressed ERα and beta (ERβ) in cultured rACC cells (Supplementary Fig. S3B and S3C).

Figure 3. Effects of E2 on glutamatergic excitatory synaptic transmission. (A) E2 dose-dependently enhances EPSCs in rACC pyramidal neurons, which is blocked by ICI 182, 780 (ICI). Representative traces are averages of 10 EPSCs. (B) E2 enhances NMDA/AMPA ratio, which is blocked by ICI. Representative traces are averages of 10 EPSCs. AMPA-EPSCs and NMDA-EPSCs in response to stimulation at holding potentials of −70 mV in the presence of APV (50 μM) and +40 mV in the presence of CNQX (20 μM), respectively. Measurements are counted at the peak point for the traces at −70 mV and at 40 ms after the stimulation artifacts for the traces at +40 mV. (C) E2 dose dependently enhances NMDAR-evoked currents. (D) Low dose of E2 decreases AMPAR-evoked currents. *\(P < 0.05\), **\(P < 0.01\) versus control; #\(P < 0.05\), ##\(P < 0.01\) versus 100 nM E2.

NMDA receptor activation by E2 was further confirmed in vivo. Following an intra-rACC injection of E2 (20 μM, 0.6 μL), phosphor-NR1 (pNR1), and phosphor-NR2B (p-NR2B) were significantly increased within 30 min (Supplementary Fig. S2A). In addition, we observed that bath application of E2 (100 nM) for 10 min produced a robust increase of pERK and pCREB in rACC slices, which was significantly blocked by the selective NMDA receptor antagonist APV (50 μM) (Supplementary Fig. S2B–E). The rapidly increased phosphorylation of ERK, CREB, NR1, and NR2B within a short time frame is consistent with rapid signaling events E2 evoked (Liu et al. 2008).
Estrogen is Sufficient to Induce NMDA Receptor-Mediated LTP via the GPCR-Associated PKA-NR2B Pathway

NMDA receptors are critical for the induction of LTP in the ACC (Zhao, Toyoda, et al. 2005). Our aforementioned data revealed that E2 rapidly enhanced the NMDA component of the EPSCs (NMDA–EPSCs), suggesting that estrogen can modulate rACC neurons' synaptic plasticity. To test this possibility, we first examined the long-term effects of E2 on NMDA–EPSCs. As expected, upon a brief application of 100 nM E2 for 6 min, the onset of NMDA–EPSCs was rapid, and long-lasting potentiation was seen for more than 40 min...
(termed NMDA-LTP) (Fig. 4A). In some neurons, which were recorded over a long period, E2-induced NMDA–LTP persisted for at least 100 min (Supplementary Fig. S4A). Similarly, a bath application of membrane-impermeable estrogen conjugate E2-BSA can also directly elicit NMDA–LTP, suggesting an effect of mERs (Fig. 4B). On the other hand, we also examined whether E2 has a long-lasting effect on AMPA–EPSCs. Different from NMDA–EPSCs, AMPA–EPSCs were obviously depressed for 20 min by a 6 min application of E2, after which, they gradually recovered to baseline (Supplementary Fig. S4B).

To determine whether E2-induced NMDA–LTP is ERs dependent, we pre-perfused ICI (1 μM) before E2 application and found that NMDA–LTP was completely blocked (Fig. 4C). It has been suggested that mERs rapidly alter cellular physiology by activating G-protein-coupled receptor (GPCR)-associated cell signaling cascades, including the activation of PKA (Hammes and Levin 2007; Tang et al. 2008). In the present study, G-protein inhibitor GDP-β-S (1 μM) in the intracellular solution completely prevented E2-induced NMDA–LTP (Fig. 4D). E2-induced NMDA–LTP was also blocked by bathing H89 (1 μM), a specific PKA inhibitor, without a change in basal NMDA–EPSCs (Fig. 4E). Furthermore, NMDAR subunit NR2B antagonist Ifenprodil (3 μM) significantly shortened the maintenance time of the NMDA–EPSC potentiation, suggesting that NR2B may contribute to the maintenance of E2-induced NMDA–LTP in the rACC (Fig. 4F).

To test the effects of endogenous estrogen on LTP of synaptic responses in the rACC, we used TBS to induce LTP. This paradigm is thought to be physiological, since the synchronized firing patterns at similar frequencies are observed during learning in the hippocampus (Otto et al. 1991). TBS produced a significant long-lasting potentiation of synaptic responses in the rACC pyramidal neurons. The amplitude of LTP was significantly suppressed by ERs antagonist ICI 182, 780 (1 μM) (Fig. 4G). To address whether TBS can elicit endogenous estrogen release, we collected perfusate of rACC slices within 5 min before TBS and 5 and 10 min after TBS to analyze for levels of E2 release. Following the TBS, E2 concentrations in perfusate were significantly increased at 10 min, compared with the 5-min pre-TBS condition (1-way ANOVA, \( F_{2,15} = 8.386, P < 0.01 \)) (Fig. 4H).

**Estrogen Rapidly Induces Formation of Dendritic Spines**

Several studies have demonstrated that estrogen treatment can increase pyramidal neuron dendritic spine density in hippocampus and cortex (Srivastava et al. 2008, 2010; Martin and Wellman 2011). We used GFP-lentivirus to transfact cultured rACC neurons. Three days after transfection, the living cells can emit steady green fluorescence upon illumination by the light of suitable wavelengths. To investigate whether estrogen could rapidly increase the connectivity of neurons, we assessed dendritic spine morphology in pyramidal neurons following treatment of 100 nM E2 for a maximum of 105 min. A significant increase in both dendritic spine and dendritic filopodia density occurred at 35 min and lasted 103 min after E2. The average spine length and number were significantly increased as compared to vehicle controls at all time points observed (2-way ANOVA, spine number: \( F_{1,42} = 5.291, P < 0.05 \); spine length: \( F_{1,42} = 10.241, P < 0.01 \)) (Fig. 5) (Supplementary Movie S1). These findings suggest that estrogen may rapidly enhance excitatory synaptic function by generating spines in rACC neurons to modulate neuronal physiology.

**Discussion**

Accumulating evidence indicates that estrogen rapidly regulates synaptic plasticity and hippocampus-dependent learning memory (Liu et al. 2008; Kramar et al. 2009). The present study further showed that E2 plays a critical role in the induction of affective pain. Formalin nociceptive stimulation induced a rapid increase in extracellular concentration of E2 in the rACC and produced CPA. Blocking ERs by an administration of ICI 182, 780 or inhibiting E2 local synthesis by androstatrienedione completely blocked F-CPA. Exogenous E2 acutely and persistently potentiated NMDA-mediated excitatory synaptic transmission in rACC slices and elicited CPA behavior in vivo. In addition, E2-induced rapid dendritic spine formation suggests that augmented synaptic connectivity may also contribute to estrogen-mediated synaptic plasticity. These results suggest that estrogen in the rACC may drive affective pain via facilitating NMDA receptor-mediated synaptic transmission.

**Nociceptor-Driven Estrogen Plays a Critical Role in Affective Pain**

Sex hormones are synthesized in the gonads, and reach the brain via the blood circulation. However, the local endogenous synthesis of estrogen can also occur in mammalian brain, because enzymes needed to synthesize E2 from androgenic and cholesterol have been demonstrated in the adult male and female rat brains (Hojo et al. 2004, 2008). The current study demonstrated that both sexes exhibited similar F-CPA scores, and blocking ERs by ICI or inhibiting endogenous E2 synthesis by ATD prevented the induction of F-CPA without sex specificity. This lack of sex specificity in F-CPA testing suggests that the brain-derived neurosteroid estrogen might be implicated in pain-related negative emotion. As a neuromodulator, estrogen can be locally synthesized and release to exert acute effects on synaptic physiology within minutes, which is faster than fluctuations in circulating estrogen (Liu et al. 2008; Srivastava et al. 2008; Smejkalova and Woolley 2010). Inhibiting the activity of aromatase, a key rate-limiting enzyme in E2 synthesis, results in a reduction of dendritic spine morphogenesis in the hippocampus (Kretz et al. 2004) and cortex (Srivastava et al. 2008). As a support, we revealed that formalin nocuous stimulation in both sexes elicited an analogous increase in extracellular E2 concentration, which was robustly suppressed by pre-treatment of ATD. More importantly, formalin-induced increase in E2 release in the rACC and F-CPA acquisition were also observed in OVX rats, providing further evidence for the brain source of E2. Intriguingly, either formalin-induced increase in extracellular E2 concentration or F-CPA score were less in OVX rats than naive adult female ones, suggesting that ovarian E2 in adult female rats was not ruled out completely in this process. Actually, it was also reported that TBS in hippocampal slices from OVX rats impaired memory (Liu et al. 2008; Kramar et al. 2009).
Systemically administered estrogen has been reported to produce dose-dependent aversive effects in both male and female, applying conditioned taste aversion (CTA) and CPA procedures in mice and rats (Miele et al. 1988; de Beun et al. 1991). However, so far, virtually less is known about the neural mechanisms mediating the aversive effects of E2 as revealed by CPA and CTA procedures. Our present study found that direct injection of E2 into the rACC rather than the PFC produced CPA, implying a critical role of the rACC in E2-induced aversive learning. Notably, formalin-induced increase in E2 release was only detected in the rACC but not the PFC, suggesting that the selective increase in E2 level in the rACC by formalin noxious stimulation might be required to acquire F-CPA. A previous study from Fields’ group demonstrated that rACC neuronal activity is sufficient to generate an aversive teaching signal, because intra-rACC injection of glutamate receptor agonist during conditioning produces robust CPA in the absence of input from primary afferent nociceptors (Johansen and Fields 2004). The ACC is a major terminus or relay site for a nociceptive afferent pathway, and nociceptive stimuli can reliably activate neurons in the ACC (Sikes and Vogt 1992). The activation of rACC neurons by noxious stimuli is necessary to produce F-CPA, and direct activation of rACC neurons is sufficient to produce aversion (Johansen et al. 2001; Johansen and Fields 2004). In the present study, we observed that E2 acutely increased the probability of calcium dependent presynaptic glutamate release (Supplementary Fig. S3) and facilitated excitatory synaptic transmission (Fig. 3). Although the evidence is not conclusive, formalin noxious stimulation might specifically trigger E2 increase in the rACC leading to the induction of pain-related aversion through NMDAR-mediated glutamatergic activation of rACC neurons.

**Rapid Potentiation of Excitatory Synaptic Transmission by Estrogen in the rACC**

Estrogen acutely potentiates excitatory synaptic transmission and facilitates the formation of LTP in the hippocampus, and...
both presynaptic and postsynaptic mechanisms are involved (Wong and Moss 1992; Neufang et al. 2009; Smejkalova and Woolley 2010). We further demonstrated that E2 rapidly potentiates excitatory synaptic transmission in rACC neurons. Significant decrease in PPR by E2 indicates an increasing probability of calcium dependent presynaptic glutamate release (Dobrunz and Stevens 1997), and enhanced NMDA-evoked current in the presence of TTX suggests an increasing postsynaptic sensitivity to glutamate. Thus, both presynaptic and postsynaptic mechanisms may also be involved in E2-induced potentiation of EPSCs in rACC neurons.

Comparing the NMDA and AMPA components of excitatory synaptic activity, we found that E2 markedly increased the ratio of NMDA transmission to AMPA transmission dose dependently. This robust increase in the NMDA/AMPA ratio is due to opposite effects of E2 on AMPA and NMDA transmission: long-term potentiation of NMDA–EPSCs and short-term depression of AMPA–EPSCs. We speculated that brief E2 might induce the generation of silent synapses by endocytosis of AMPAR in rACC neurons, which results in AMPA transmission transiently decreased, and then, reinstated via AMPAR trafficking back to synaptic area from extrasynaptic area. As an anatomical support, Srivastava et al. demonstrated that E2 transiently increased synaptic NMDAR subunit NR1 and removed AMPAR subunit GluR1 from the surface into dendritics shafts. Consistent with this trafficking, a transient decrease in AMPAR mediated transmission was observed after 30 min, but not after 60 min, of treatment with E2 (Srivastava et al. 2008). This bidirectional pattern of NMDAR and AMPAR redistribution is consistent with the generation of silent synapses (Kim et al. 2003; Marie et al. 2005). These functional silent synapses can be activated through GluR1 insertion into synapses lacking this subunit after an LTP-inducing protocol (Liao et al. 2001; Lu et al. 2001). It has been revealed that NMDAR activation after E2 treatment resulted in significantly higher surface GluR1 levels than in controls and after E2 treatment per se, suggesting that the silent synapses induced by E2 could be converted to active synapses by subsequent NMDAR activation (Srivastava et al. 2008).

It should be mentioned that in the present study, E2 decreased AMPA-evoked currents only at low dose (10 nM) but not at 100 nM (Fig. 3D). Although the divergent effects is currently unclear, it is possible that the disparate effects are caused by differential binding kinetics of different doses of E2 to different ERs that may be coupled to various signaling events. For instance, E2 stimulation of membrane-localized ERα was found to trigger mGlu1a signaling, leading to the activation of Gq-mediated stimulation of PLC, PKC, and IP3 signaling, and eventual MAPK-dependent CREB phosphorylation. However, in the same population of neurons, membrane-localized ERα and ERβ could also trigger activation of mGlu2, leading to Gi/o-coupled decreases in cAMP and subsequent attenuation of L-type calcium channel-dependent CREB phosphorylation (Boulware et al. 2005; Srivastava et al. 2011). Further studies will need to be performed to understand the contribution of the different ERs.

**Membrane ERs-Initiated, G-Protein-Dependent Rapid Signaling Pathway**

Estrogen rapidly regulates cellular physiology via membrane-initiated, G-protein-dependent rapid signaling pathways, including kinases, ion channels and second messengers (Ivanova et al. 2002; Wu et al. 2005; Zhao, Chen et al. 2005).

In support of these, we observed that E2-BSA mimicked E2-induced CPA and LTP of NMDA–EPSCs, which was completely blocked by GPCR inhibitor GDP-β-S and ICI 182, 780 that has been reported to inhibit cytoplasmic ERs (Kalita et al. 2005; Jelks et al. 2007). These results suggest that E2-induced acute action on rACC synaptic transmission is, at least in part, mediated by G protein-coupled mER (i.e. GPR30) or classical ERα and ERβ that directly bind to G proteins or link to other G proteins-coupled receptors (e.g. mGluRs) that then induce signaling pathways. We found that E2-induced NMDA–LTP in the rACC was completely blocked by a PKA inhibitor, providing further support for regulation of NMDAR activity by mERs–PKA. The crosstalk between ERs, PKA and NMDAR has been involved in spinal nociceptive modulation. PKA activation induces phosphorylation of NMDAR subunits and PKA inhibitors attenuate NR1 phosphorylation in the spinal cord (Tang et al. 2008). In this study, we observed rapid NMDAR subunits NR1 and NR2B phosphorylation after intra-rACC application of E2 in vivo. Also, E2-induced NMDA–LTP in the rACC was markedly suppressed by an NR2B antagonist. It is plausible that E2 activates PKA by G protein signaling, and then phosphorylates NMDAR (Fig. 6).

On the other hand, several sources of evidence suggest that NMDA receptors are closely involved in mechanisms associated with E2-induced glutamate synapse formation in the hippocampus (Woolley and McEwen 1994; Morissette et al. 2008). E2 can induce an enrichment of the NR1 subunit of NMDA receptors in synaptic clusters and is linked with an increase of ERα-immunoreactive clusters along the dendrites (Jelks et al. 2007). In the present study, we observed wide colocalization of ERα, ERβ, or GPR30 with NMDAR subunit NR1 and rapidly enhanced NMDA-evoked current by E2 in...
the rACC. Ca\(^{2+}\) influx via NMDAR can elevate intracellular cAMP levels leading to the activation of PKA. PKA then activates ERK pathway through Rap1, then B-Raf (Sindreu et al. 2007). NMDAR NR2B has also been reported to directly couple to the ERK pathway via Ras-GRF1 (Krapivinsky et al. 2003). Our previous study revealed that NMDA induced a robust pERK increase and formalin-induced pERK could be completely blocked by APV in the rACC (Cao, Cui, et al. 2009). Here, we further showed that E2 rapidly phosphorylated ERK and CREB and which was blocked by APV. Together with our previous report that the activation of NMDAR–PKA–ERK–CREB pathway in the rACC is required for the induction of pain-related negative affect, it is also reasonable to believe that estrogen contribute to pain-related aversion via mERs–NMDAR–PKA–ERK–CREB pathway-mediated rapid action (Fig. 6).

**Dendritic Spine Morphogenesis, Synaptic Plasticity and Pain-Related Aversive Behavior**

The formation of dendritic spines is a well-defined representation of cell-to-cell communication (Alvarez and Sabatini 2007). Changes in dendritic spine number, morphology and axonal outgrowth are important signifiers of synaptic plasticity and altered neuronal circuitry. In the present study, E2 not only increased the dendritic spine number and length, but also affected the growth of filopodia. Dendritic filopodia serves primarily to initiate synaptogenic contacts with nearby axons and acts as a first step in its transformation into a stable dendritic spine (Ziv and Smith 1996; Coccurello et al. 2012). The density of excitatory glutamate synapses are positively correlated with enhanced learning memory. Memory can be enhanced during the time frame when estrogen induced an increase in synapse density (Leuner et al. 2003; Tang et al. 2004). E2-induced increase in hippocampal LTP magnitude only occurs when spine density is increased simultaneously with an escalation in NMDA transmission relative to AMPA transmission (Smith and McMahon 2005). Given that synapse density, NMDA receptor function and hippocampal LTP are associated with learning and memory, it is likely that estrogen modulates these parameters to facilitate the observed improvement in learning memory. Recently, Srivastava et al. reported a mechanism for 2-step wiring plasticity, whereby an ER\(\beta\)-mediated increase in synaptic connectivity by estrogen is sustained by subsequent NMDA receptor activation (Srivastava et al. 2008). Moreover, the activation of ER\(\beta\) increased dendritic branching and density of mushroom-type spine of hippocampal neurons, also enhanced LTP magnitude (Liu et al. 2008). In the present study, we revealed that E2 rapidly and persistently potentiated NMDA transmission, increased pERK and pCREB, a process which has been strongly implicated in the formation of LTP in the hippocampus and rACC (Lonze and Ginty 2002; Toyoda et al. 2007; Cao, Cui, et al. 2009), remodeling of dendritic spines (Shansky et al. 2010; Srivastava et al. 2010) and pain-related aversion (Cao, Gao, et al. 2009). We also observed a pronounced increase in density and length of the spine upon a treatment of E2. Blocking ERs prevented E2-induced NMDA–LTP, suppressed TBS-induced LTP amplitude and impaired pain-related aversive acquisition. As we all know that NMDA receptor activation is the main approach to increasing intracellular calcium concentration and nuclear signals (e.g. CREB), activated by an increase in Ca\(^{2+}\), are involved in the central regulation of spine formation and memory learning (Segal 2001; Okada et al. 2003). Furthermore, E2 enhances the density of dendritic spines are involved in neuronal synaptic connection by induced Ca\(^{2+}\) influx and CREB activation (Zhao, Chen et al. 2005).

Together, all the data suggest a tight mechanistic coupling between these morphological and functional changes. Estrogen, as a neuromodulator in the rACC, may drive affective pain via facilitating NMDA receptor-mediated synaptic transmission and plasticity (Fig. 6). More research is needed to further elucidate the role of estrogen on the mechanistic underpinnings of rACC activation and affective pain. After figuring out which of the primary ER\(\beta\)s are involved in and confirming the clear signaling pathways, a new strategy targeted at brain-derived neurosteroids and relative receptors might be raised for the prevention of pain-related emotional disturbance in both sexes.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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