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Regular Aerobic Exercise Attenuates Pain and Anxiety in Mice by Restoring Serotonin-Modulated Synaptic Plasticity in the Anterior Cingulate Cortex

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BASIC SCIENCES

ABSTRACT

ZHOU, Y.-S., F.-C. MENG, Y. CUI, Y.-L. XIONG, X.-Y. LI, F.-B. MENG, Z.-X. NIU, J.-X. ZHENG, Y.-Q. QUAN, S.-X. WU, Y. HAN, and H. XU. Regular Aerobic Exercise Attenuates Pain and Anxiety in Mice by Restoring Serotonin-Modulated Synaptic Plasticity in the Anterior Cingulate Cortex. *Med. Sci. Sports Exerc.*, Vol. 54, No. 4, pp. 566–581, 2022. **Purpose:** Clinical studies found that regular aerobic exercise has analgesic and antianxiety effects; however, the underlying neural mechanisms remain unclear. Multiple studies have suggested that regular aerobic exercise may exert brain-protective effects by promoting the release of serotonin, which may be a pain modulator. Anterior cingulate cortex (ACC) is a key brain area for pain information processing, receiving dense serotonergic innervation. As a result, we hypothesized that exercise may increase the release of serotonin in the ACC, thus improving pain and anxiety behaviors. **Methods:** Integrative methods were used, including behavioral, electrophysiological, pharmacological, biochemical, and genetic approaches, to explore the effects of regular aerobic exercise and the underlying neural mechanisms. **Results:** Regular aerobic exercise in the form of voluntary wheel running for 30 min daily for 15 d showed significant effectiveness in relieving pain and concomitant anxiety in complete Freund's adjuvant–induced chronic inflammation pain models. c-Fos staining and multielectrode array recordings revealed alterations in neuronal activities and synaptic plasticity in the ACC. Moreover, systemic pharmacological treatment with 4-chloro-DL-phenylalanine (PCPA) to deplete endogenous serotonin and local delivery of serotonin to the ACC revealed that exercise-related serotonin release in the ACC bidirectionally modulates pain sensitization and anxiety behaviors by modulating synaptic plasticity in the ACC. Furthermore, we found that 5-HT1A and 5-HT7 receptors mediated the serotonin modulation effects under conditions of regular aerobic exercise through local infusion of a selective antagonist and shRNA in the ACC. **Conclusions:** Our results reveal that regular aerobic exercise can increase serotonin release and modulate synaptic plasticity in the ACC, ultimately improving pain and concomitant anxiety behaviors through the functions of the 5-HT1A and 5-HT7 receptors. **Key Words:** PAIN, ANXIETY, AEROBIC EXERCISE, SEROTONIN, 5-HT1A, 5-HT7

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Exercise is fundamental for good health. Previous studies demonstrated that regular physical activity could mitigate chronic pain in human (1,2). For example, the frequency, intensity, and duration of migraines are significantly reduced through bike training regularly (2). Furthermore, prescribed exercise is an efficacious method for an array of pain conditions in clinical practice (3). Recent year, many animal researches have focused on the underlying mechanisms of physical activity on ameliorated pain condition. Substantial studies show that the expression of the serotonin transporter and phosphorylation of the NMDA receptor in the rostral ventromedial medulla were increased in animals with chronic pain and that upregulation was diminished in physical active animals (4,5). However, the mechanism beneath analgesia effect of physical activity in the higher level of central nervous system remains unclear. Recently, cumulative animal studies support the idea that regular aerobic exercise can activate the dorsal raphe

nucleus and promote the release of endogenous serotonin (6,7), which may contribute to exercise-related brain protection effects. Furthermore, it has been demonstrated that increased serotonin in some certain brain areas has the analgesia effect in rodent researches (8,9). It is indicated that physical activity may exert its beneficial effect on pain behavior in animal studies through the increased release of serotonin in the brain. However, whether physical activity exerts analgesic effect through increased endogenous serotonin release and whether the target is corresponding to the elevated serotonin are still unclear.

The anterior cingulate cortex (ACC) is known to regulate fundamental cognitive processes. Abundant evidence from both humans and animals demonstrates that forebrain cortical areas, including the ACC, are activated by various acute somatic and visceral noxious stimuli and are important for pain and pain-related perception and regulation (10). Cellular and molecular studies using rodent models have provided insights into the mechanisms involving the ACC, which leads to and sustain chronic pain and anxiety (11). Recent studies have demonstrated that exogenous administration of serotonin in certain brain areas can produce analgesic effects, suggesting that serotonin in the brain plays an important role in the regulation of pain (8,9). Furthermore, the ACC neurons are highly innervated by serotoninergic terminals (12). Research has found that the activation of 5-HT7 receptor inhibits mechanical allodynia in nerve-injured animals by affecting dendritic function in the ACC (13). Another study has found that downregulation of the 5-HT1A receptor in the ACC is involved in stress-induced visceral hyperalgesia and contributes to anxiety-like behavior (14). In addition, inhibition of the 5-HT1A receptor could increase presynaptic glutamate release in the ACC (15). The above mentioned evidence highlights the vital roles of serotonin and its receptors in the ACC in the processing of chronic pain and concomitant anxiety. However, whether the changes in serotonin release caused by regular aerobic exercise are involved in pain-related plasticity in the ACC and whether they mediate the mechanism of exercise-related pain improvement remain unclear.

In this study, we aimed to test the hypothesis that wheel running increases serotonin release and modulate synaptic plasticity in the ACC, ultimately improving pain and concomitant anxiety behaviors through the functions of the 5-HT1A and 5-HT7 receptors.

METHODS

Experimental animals. Adult male C57BL/6J mice were used in this study. All mice were housed in groups of 4 to 6 individuals per cage (individually ventilated cages) with access to food and water *ad libitum*. The room temperature and relative humidity were maintained at 22°C to 25°C and 40% to 60%, respectively. After surgery for cannula implantation and microinjections of virus, the mice were allowed to recover for 7 d before drug delivery and were allowed 21 d for virus expression. All experiments were approved by the Institutional Animal Care and Use Committee of AFMU, and the animals were maintained in accordance with the guidelines set forth by the International Association for the Study of Pain. All efforts were made to minimize the number of animals used in this study in line with 3R core principles. To induce inflammatory pain, 15 μL of complete Freund's adjuvant (CFA) (F5881; Sigma) was injected subcutaneously into the left hindpaw according to previous studies (16,17).

Regular aerobic exercise. Previous animal studies have demonstrated that various forms of exercise, including swimming, treadmill training, and wheel running, can relieve pain (18–20). Among those exercise forms, behavioral paradigms can be roughly divided into forced and free movements. However, they both have their own shortcomings. For example, forced exercise causes greater stress to animals and requires a longer adaptation period. With free access, it is difficult to monitor and control the amount of exercise. Therefore, we designed a modified protocol to mimic the clinical prescription of regular exercise and minimize the stress induced by the training method and external environments. We used a modified training paradigm with a homemade enclosed running wheel to explore the effects of exercise on inflammatory pain and concomitant anxiety during an 18-d experimental course (Fig. 1A). When we put the mouse into the enclosed wheel, the mouse could run or stop with its own driving force. In contrast to forced treadmill exercise, the animals were allowed to run in a semivoluntary manner for 30 $min \cdot d^{-1}$ for 15 d. Before training, the mice were transferred to a dimly lit room and allowed to adapt to the environment for at least 30 min. Then, each animal was gently transferred into a running wheel, and the total distance run was monitored by an infrared high-definition camera (Sony, SNC-VB642D). At the end of the training, the mice were gently brought back to their home cages. The running wheel was cleaned with double-distilled water and 75% ethanol to remove any possible olfactory cues between each animal. All efforts were made to reduce the stress caused by the external environment.

Allodynia and hyperalgesia behavior assessments. Mechanical allodynia was assessed as reported previously (21) using von Frey filaments (Stoelting Corporation, USA). The tests were carried out during light cycle. The animals were handled and allowed to adapt to the behavioral test environment for 1 wk before testing. The mice were placed in a cubic plastic container with a wire mesh floor and allowed to acclimate for 30 min before testing. Mechanical allodynia was assessed based on the responsiveness of the hindpaw using von Frey filaments with different bending forces (0.008*g*–2*g*). Positive responses include licking, biting, and sudden withdrawal of the hindpaw. Each filament was applied 5 times with an interval of 5 min, and the paw withdrawal threshold (PWT) was defined as the minimum force for three positive responses. Then, the mice were allowed to rest for 30 min in their home cage. Next, hyperalgesia was tested on a warmed transparent Perspex sheet after an adaptation duration of 30 min. Sensitivity to painful heat was measured by means of a Hargreaves test (IITC Life Science, USA). The hindpaw plantar surface was exposed to a laser beam until paw withdrawal, and its latency was recorded. The mean paw withdrawal latency (PWL) in each of the five tests with at least

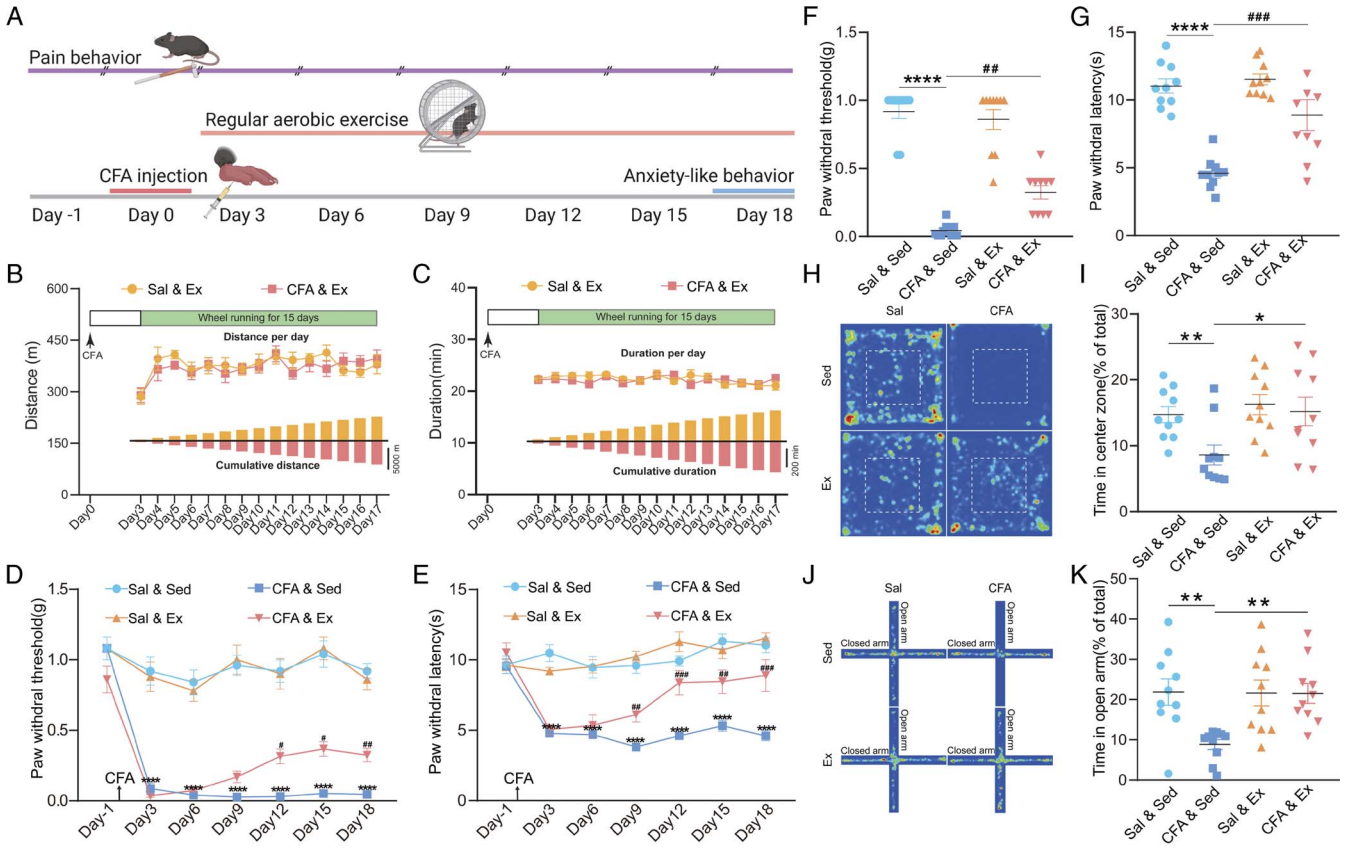

FIGURE 1—Aerobic voluntary wheel running attenuates sensory and affective components of inflammatory pain. A, Experimental schematic. B, C, Comparison of wheel running distance (B) and duration (C) over 30 min between the saline and exercise group (Sal & Ex) and the CFA and exercise group (CFA & Ex). *n* = 10. D, E, Effect of exercise on mechanical allodynia during the time course (D) and at day 18 (E). *n* = 10. F, G, Effect of exercise on thermal hyperalgesia during the time course (F) and at day 18 (G). *n* = 10. Among the saline and sedentary group (Sal & Sed), the CFA and sedentary group (CFA & Sed), the saline and exercise group (Sal & Ex), and the CFA and exercise group (CFA & Ex), an asterisk (*) indicates that the Sal & Sed group was compared with the CFA & Sed group, and a crosshatch (#) indicates that the CFA & Sed group was compared with the CFA & Ex group. (H, I) Typical trace (H) and cartogram (I) from the open field test. Dotted box, center zone of the open field. *n* = 10. (J, K) Typical trace (J) and cartogram (K) from the elevated plus maze test. *n* = 10. Among the typical traces (H, J), the saline and sedentary group (Sal & Sed) trace is shown in the upper left, the CFA and sedentary group (CFA & Sed) trace is shown in the upper right, the saline and exercise group (Sal & Ex) is shown in the lower left, and the CFA and exercise group (CFA & Ex) is shown in the lower right. Repeated one-way ANOVA followed by Tukey's multiple comparisons test was used to compare the time points (D–G, *****P* < 0.0001; ###*P* < 0.001, ##*P* < 0.01, #*P* < 0.05), and Kruskal-Wallis H test followed by Dunn's multiple comparisons test was used to compare the Sal & Sed group with the CFA & Sed groups and the CFA & Sed group with the CFA & Ex group (I, K, ***P* < 0.01, **P* < 0.05). The data are presented as the mean ± SEM.

10-min intervals was addressed for statistical analysis. All behavioral tests were performed in a double-blind trial fashion.

Elevated plus maze test. The maze apparatus consisted of two open arms and two enclosed arms (60 cm × 60 cm) situated perpendicular to each other. During the trial, the subjects were placed in the center square of the maze, facing an open arm. Then, the behavior of the animal was monitored for 5 min with a camera (Sony, SNC-VB642D) fixed above the maze. To reduce the variability and increase the reliability, we performed the behavior test during the light cycle according to previous study (22). The time spent in each arm and the total distance traveled were subjected to further analysis with a video tracking system (Smart 3.0, Panlab, S.L., Spain). The elevated plus maze was cleaned with 75% ethanol after each mouse was tested to remove any possible olfactory cues. All behavioral tests were performed in a blinded trial fashion, which refers to the fact that we kept the experimenters performed behavioral test and data analysis both blinded to the groups of the animals.

Open field test. The open field chamber was made of a black box with a square white plastic bottom (50 × 50 cm). Before the start of the test, the field was thoroughly washed with detergent, double distilled water and 75% ethanol to remove any possible olfactory cues. At the beginning of the trial, each mouse was placed in the center of the chamber with dim illumination, and activity was measured for 10 min with an overhead video-tracking system (Smart 3.0, Panlab, S.L., Spain). At the end of the experiment, the percentage of time the subject spent in the center area and the total distance traveled were analyzed. All behavioral tests were performed in a double-blind trial fashion.

Immunohistochemistry. c-Fos immunohistochemistry staining was used to detect behavior-related neuronal activity in mice. At designated time points, animals were anesthetized and perfused through the aortic ventricle with 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the brains were removed, postfixed (in the same fixative) for 2 h and then placed in 30% sucrose

for 3 d. After dehydration, frozen brain tissue containing the ACC underwent coronal sectioning (35 μm thickness) on a freezing microtome (Leica). The sections were immunostained for c-Fos protein with the avidin–biotin–peroxidase complex method. The slices containing the ACC were rinsed three times in 0.01 M phosphate-buffered saline (PBS) and then incubated with a solution containing 2.5% Triton X-100 and 3% goat serum for 1 h at room temperature. The sections were further incubated with a polyclonal antibody raised in rabbits against *c-Fos* (1:3000; 2250S; Cell Signaling Technology) for 24 h at 4°C and then incubated with biotinylated goat antirabbit IgG (1:200; ZSGB-Bio) and avidin-biotin-peroxidase complex (1:200; ZSGB-Bio). The reaction product was visualized with 0.01% hydrogen peroxide and 0.05% diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.6). Between incubations, the sections were rinsed three times in 0.01 M PBS for 10 min per wash. Images were obtained using a microscope attached to a CCD spot camera (Olympus, Japan) and processed with ImageJ software.

Immunofluorescence. At designated time points, animals were anesthetized and perfused through the aortic ventricle with 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the brains were removed and postfixed (in the same fixative) for 2 h and then placed in 30% sucrose for 3 d. After dehydration, frozen brain tissue containing the ACC underwent coronal sectioning (35 μm thickness) on a freezing microtome (Leica). The sections were rinsed three times in 0.01 M PBS for 10 min per wash and incubated with DAPI fluorescent dye (R37606; 2 drops/mL; Invitrogen) for 20 min. Then, the slices containing the ACC were rinsed three times in 0.01 M PBS and mounted onto microscope slides. Images were obtained using a confocal microscope attached to a CCD spot camera (FV3000; Olympus, Japan).

Enzyme-linked immunosorbent assay. To investigate whether serotonin release was altered in the ACC with regular aerobic exercise training, serotonin levels in the ACC were tested by enzyme-linked immunosorbent assay. The levels of serotonin in the ACC were determined 15 d after regular aerobic exercise via enzyme-linked immunosorbent assay according to the manufacturer's instructions. Briefly, the bilateral ACC was dissected from the brain and homogenized with ice-cold sterile 0.1 M PBS via ultrasonication. The supernatant was collected, and the amount of 5-HT in each sample was analyzed in duplicate using an enzyme-linked immunosorbent assay kit (E-EL-0033c, Elabscience, China).

Western blot analysis. Western blot analysis was performed as described previously (23). The bilateral ACC was dissected from the brain and homogenized with RIPA buffer containing a protease inhibitor cocktail (Roche Diagnostics) on ice. After centrifugation at 12,000*g* for 15 min at 4°C, the total protein content of the supernatant was quantified using a bicinchoninic acid protein assay (Applygen Technologies, Inc.). Twenty microgram protein per well was loaded. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Invitrogen). Primary antibodies against the following targets were used: the 5-HT1A receptor (1:100; 24504; ImmunoStar) and the 5-HT7 receptor (1:100; 24430; ImmunoStar). The proteins were visualized using the enhanced chemiluminescence detection method (Advansta). ImageJ version 2.1.0 software (National Institutes of Health) was used to measure protein band intensity. To guarantee imaging was in the linear range, concentration of each protein sample was homogenized. High-concentration protein samples were diluted and adjusted to 1 $mg \cdot mL^{-1}$. Exposure time was controlled. To normalize blots between gels, all the factors stated above were tried to keep the same. Meanwhile, housekeeping protein "beta-actin" was detected on the same blot as the target protein for each gel. The ratio of target protein to internal (dividing) was calculated, and different gels were put together for normalized analysis.

Cannula implantation and microinjection procedures. Mice were anesthetized with isoflurane at a flow rate of 0.3 $L \cdot min^{-1}$ and placed in a stereotaxic instrument (RWD Life Science, Ltd., China). The fur on the skull was shaved, and the skin was cleaned with iodophor three times. Ointment was applied to the eyes to prevent corneal damage. An incision was made over the skull, and the surface was exposed. A 26-gauge guide cannula was implanted bilaterally into the ACC. The stereotaxic coordinates of the ACC were anterior/posterior +0.5 mm, medial/lateral ±0.25 mm, and dorsal/ventral −1.5 mm. The microinjection cannulas were secured to the skull with dental cement. The mice were injected with 0.2 mL of sterile saline and antibiotic (i.p.) for hydration and prevention of postoperative infection. The mice were given 1 wk to recover after cannula implantation. Drugs were delivered 30 min before wheel running exercise. The mice were anesthetized with isoflurane at a flow rate of 0.3 $L \cdot min^{-1}$. Sterile 33-gauge microinjection needles were connected to two 1-μL syringes (Hamilton) via microbore tubing. The syringes were mounted on a microinfusion pump (RWD Life Science, Ltd., China) set to deliver fluids at a flow rate of 150 $nL \cdot min^{-1}$. The microinjection needles were inserted bilaterally to a depth of 0.25 mm beyond the ventral tip of the guide cannula. The drug solutions were infused in a volume of 300 nL per side over a 2-min period. Then, the microinjection needles were left in place for an additional 2 min for drug diffusion. The injection sites were confirmed at the end of all experiments, and mice with sites outside of the ACC region were excluded from the study.

Stereotaxic virus microinjection. Similarly, mice were anesthetized with isoflurane at a flow rate of 0.3 $L \cdot min^{-1}$ and placed in a stereotaxic instrument (RWD Life Science, Ltd., China). The fur on the skull was shaved, and the skin was cleaned with iodophor three times. Ointment was applied to the eyes to prevent corneal damage. An incision was made over the skull, and the surface was exposed. A 30-gauge needle was lowered into the ACC (anterior/posterior +0.5 mm, medial/lateral ±0.25 mm, and dorsal/ventral −1.75 mm), and adeno-associated virus was injected at a flow rate of 150 $nL \cdot min^{-1}$ for 2 min per side. Then, the microinjection needles were left in place for an additional 10 min for virus

diffusion, and the skin above the skull was closed with silk suture. The mice were injected with 0.2 mL of sterile saline and antibiotic (i.p.) for hydration and prevention of postoperative infection. The mice injected with virus were allowed to recover for 3 wk before the behavioral experiments. The injection sites were confirmed at the end of all experiments, and mice with sites outside of the ACC region were excluded from the study.

ACC slice preparation. Adult male mice were anesthetized with isoflurane, and the whole brain was quickly removed from the skull and submerged in ice-cold oxygenated (95% O_2 and 5% CO_2) artificial cerebral spinal fluid (ACSF) containing the following (in mM): 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 25 $NaHCO_3$, 1 NaH_2PO_4, and 10 glucose, pH 7.35 to 7.45. Coronal brain slices (300 μm) containing the ACC were prepared with a vibrating tissue slicer (VT1200S, Leica). After cutting, the slices were incubated in a submerged recovery chamber with ACSF for at least 2 h at room temperature.

Preparation of the multielectrode Array. A MED64 probe (P515A, Panasonic) was prepared with a standard protocol (24). Before use, the surface of the MED64 probe was treated with 0.1% polyethyleneimine (P-3143, Sigma-Aldrich) in 25 mmol·L^{-1} borate buffer, pH 8.4, overnight at room temperature. Then, the probe surface was rinsed at least three times with sterile distilled water, and the probe was stored in a 4°C refrigerator.

Field potential recording. The methods for field potential recording were similar to those described previously (25). After incubation, one slice was transferred to the recording chamber, perfused with ACSF at 28°C to 30°C and maintained at a 2-mL·min^{-1} flow rate. The slice was positioned on the MED64 probe in such a way that the different layers of the ACC were entirely covered by the whole array of electrodes (Fig. 2G), and then a fine-mesh anchor was placed on the slice to ensure its stability during the experiments. After at least a 1-h recovery period for the slice in the recording chamber, biphasic constant-current pulse stimulation (0.2 ms) was applied to the stimulation channel, which was located in layer V/VI, to evoke the field excitatory postsynaptic potential (fEPSP) in the channels closest to the stimulation site. Stable baseline responses were recorded for 0.5 h, and then we used a well-established pain related theta-burst stimulation (TBS) protocol (five trains of bursts with four pulses at 100 Hz, at 200 ms intervals, repeated five times at intervals of 10 s) to induce LTP (26).

Drugs and virus. The chemicals and virus used in this study were procured from various sources. Serotonin (S4244; Selleck, USA), 4-chloro-DL-phenylalanine (PCPA) (S4586; Selleck, USA), WAY100635 (S2663; Selleck, USA), and SB269970 (S2849; Selleck, USA) were purchased from Selleck. The concentrations of these drugs and vehicle for cannular delivery in the present study were in accordance with those described previously (27–30). A *c-Fos* (1:3000; 2250S; Cell Signaling Technology) antibody was purchased from Cell Signaling Technology. DAPI (R37606; 2 drops/mL; Invitrogen) was purchased from Invitrogen. Antibodies against the 5-HT1A receptor (1:100; 24504; ImmunoStar) and the 5-HT7 receptor (1:100; 24430; ImmunoStar) were purchased from ImmunoStar. shRNAs targeting the mRNA sequences of the 5-HT1A receptor (Htr1a) and the 5-HT7 receptor (Htr7) was designed. Recombinant adeno-associated virus shRNA-expressing vectors containing Htr1a shRNA (AAV-CaMKIIa-EGFP-miR30shRNA (Htr1a)-WPRE, 5′CCCTTCAGAATGTTGCCAA3′) and Htr7 shRNA (AAV-CaMKIIa-EGFP-miR30shRNA (Htr7)-WPRE, 5′GGAAGAGTGTGCGAACCTT3′) or nonspecific control (NC) shRNA (AAV-CaMKIIa-miR30shRNA (NC)-WPRE) were constructed by Obio Technology (Shanghai Corp., Ltd.).

Experimental designs for manipulation of serotonin and its receptors *in vivo*. The PCPA treatment experiment was designed to determine whether increased serotonin in the ACC after exercise functions to rescue LTP and relieve pain and anxiety (Fig. 3B). Serotonin in the ACC was depleted by five consecutive pretreatments of parachlorophenylalanine (PCPA, 300 mg·kg^{-1}·d^{-1}) as previously described (28). Next, the subjects were subdivided into PCPA and vehicle groups. All of them were injected with CFA and trained with wheel running.

Serotonin local infusion was to test whether the increased serotonin in the ACC was related to the improvement of pain and concomitant anxiety induced by exercise. We implanted cannulas in the ACC bilaterally to deliver serotonin (Fig. 4A). Similar to the previous experimental design, we performed pharmacological experiments to locally deliver serotonin into the ACC and explored the effect of exogenous serotonin on inflammatory pain (Fig. 4B). After a 7-d recovery period, the mice were tested for basal mechanical and thermal pain. Then, CFA was injected into the left hind paw to induce inflammatory pain. After a 3-d pain behavior test, serotonin (3.5 μg) or vehicle (saline) was infused locally into the bilateral ACC in a volume of 300 nL per side (the doses were chosen according to a previous study) (27). After 30 min of drug infusion, the mice were transferred to a locked running wheel that could not rotate for 30 min. Drug was delivered and access to the locked running wheel was permitted daily during postoperative days 3 to 18 after the behavior test.

The antagonist of 5-HT1A receptor (WAY100635) and the antagonist of the 5-HT7 receptor (SB269970) were used to test whether 5-HT1A and 5-HT7 receptors in the ACC were involved in the rescue effects of exercise (Figs. 5A–B and Figs. 7A–B). We implanted cannulas in the ACC to deliver the drugs and the experimental design was in accordance with the abovementioned pharmacological assay. WAY100635 (1 nM), SB269970 (100 μM) or vehicle (saline) was bilaterally infused into the ACC in a volume of 300 nL per side according to a previous study (29,30).

Viral vectors carrying shRNA were used to further verify the pharmacological discovery. Recombinant adeno-associated virus shRNA-expressing vectors containing Htr1a shRNA (AAV-CaMKIIa-EGFP-miR30shRNA (Htr1a)-WPRE), Htr7 shRNA (AAV-CaMKIIa-EGFP-miR30shRNA (Htr7)-WPRE) were injected bilaterally into the ACC at 300 nL per side to knock down the 5-HT1a or 5-HT7 receptor. Nonspecific control shRNA (AAV-CaMKIIa-miR30shRNA (NC)-WPRE) served as the control. After a 3-wk recovery period, CFA was injected into the left hind paw to induce inflammatory pain. Exercise started at day 3 and lasted until the end of the experiment.

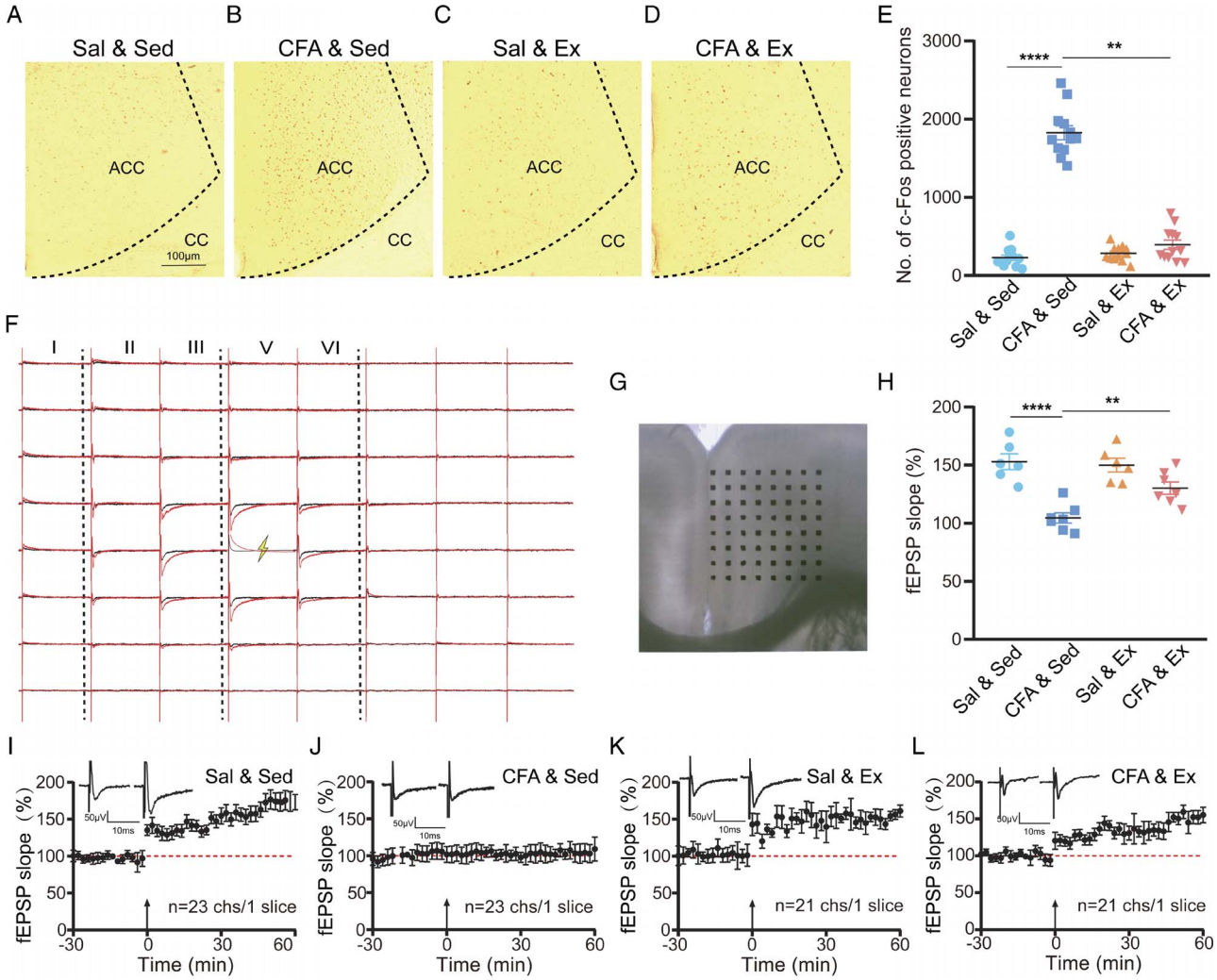

FIGURE 2—Aerobic voluntary wheel running rescues pain-induced occlusion of long-term potentiation in ACC slices. A–D, Typical immunohistochemistry images of *c-Fos* staining among the saline and sedentary group (A, Sal & Sed), the CFA and sedentary group (B, CFA & Sed), the saline and exercise group (C, Sal & Ex), and the CFA and exercise group (D, CFA & Ex). E, Statistical chart of *c-Fos* staining. *n* = 12 slice from four mice. F, G, Typical images of long-term potentiation (F) and electrode position (G) in the MED64 recording system. Lightning symbol, stimulation site; black line, fEPSP before TBS; red line, fEPSP after TBS; Roman numerals, ACC layers (F). H–L, Statistical chart of MED64 recordings of the fEPSP slope during the last 10 min (H). *n* = 6–7 slice. Typical images of MED64 recordings of fEPSP slopes among the saline and sedentary group (I, Sal & Sed), the CFA and sedentary group (J, CFA & Sed), the saline and exercise group (K, Sal & Ex), and the CFA and exercise group (L, CFA & Ex). Arrows, TBS (I–L). Kruskal-Wallis H test followed by Dunn's multiple comparisons test was used to compare the Sal & Sed group with the CFA & Sed groups and the CFA & Sed group with the CFA & Ex group (E, ***$P < 0.001$, **$P < 0.01$). One-way ANOVA followed by Bonferroni's multiple comparison test was performed between the Sal & Sed and CFA & Sed groups and the CFA & Sed and CFA & Ex groups (H, ***$P < 0.001$, **$P < 0.01$). The data are presented as the mean ± SEM.

Mechanical allodynia, thermal hyperalgesia, and anxiety-like behavior were tested during the experimental period (Figs. 6A and 8A). The injection sites were confirmed at the end of the experiments (Figs. 6B and 8B).

Statistical analysis. The numbers of mice used in this study were selected based upon the statistical calculation on estimated sample size (see Table, Supplemental Digital Content 1, Statistical details for sample size estimation, http://links.lww.com/MSS/C460). The efficiency of statistics analysis, feasibility of experimental design, the sample size of pioneering relevant studies, and the animal welfare (3R principle) were also concerned to choose the sample size. PASS software (v15) was used to calculate the sample size.

The data are presented as the mean ± SEM. Statistical significance was measured using Student's *t* test or one-way and Repeated One-way ANOVA. Normality and homoscedasticity have been tested before using above utilized tests. SPSS software (v23) was used to calculate the normality and homoscedasticity test. The work flow of choosing appropriate statistical method was shown in Supplemental Table 2 and Supplemental Table 3 (see Table, Supplemental Digital Content 2, Summary of statistical methods, http://links.lww.com/MSS/C461; and see Table, Supplemental Digital Content 3, Statistical data for all figures and supplementary figures, http://links.lww.com/MSS/C462). A *P* value less than 0.05 was considered to indicate statistical significance. All data analyses

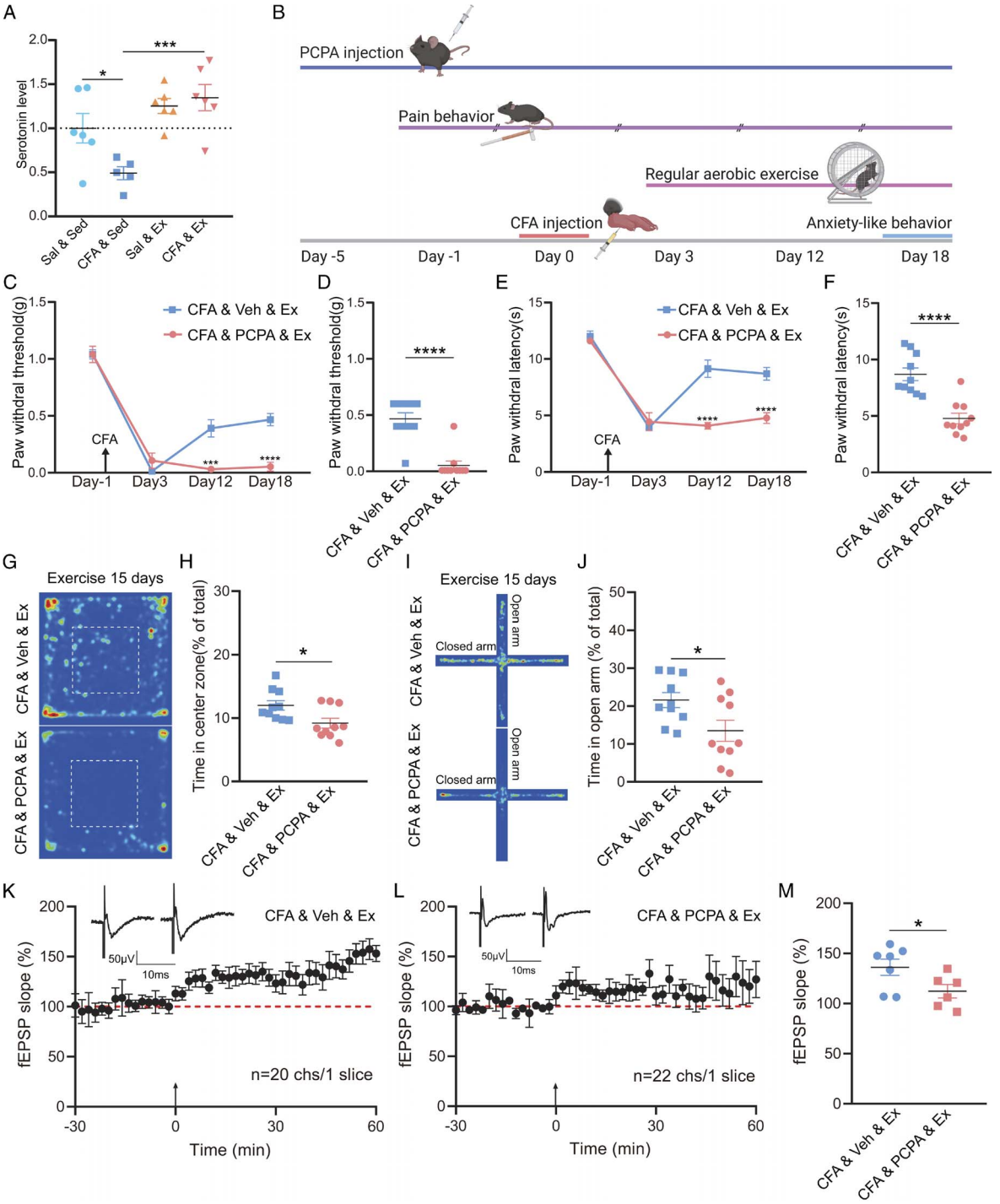

FIGURE 3—Serotonin release in the ACC allows exercise to alleviate pain and restore long-term potentiation. A, Enzyme-linked immunosorbent assay to detect serotonin levels in the ACC among the saline and sedentary group (Sal & Sed), the CFA and sedentary group (CFA & Sed), the saline and exercise group (Sal & Ex), and the CFA and exercise group (CFA & Ex). *n* = 6. B, Experimental schematic. Injection of 300 mg·kg^{-1}·d^{-1} PCPA or vehicle (saline) for five consecutive days before the behavioral test and until the end of the experiment. C, D, Effect of PCPA on mechanical allodynia during the time course (C) and at day 18 (D). *n* = 10. E, F, Effect of PCPA on thermal hyperalgesia during the time course (E) and at day 18 (F). *n* = 10. G, H, Typical trace (G) and cartogram from the open field test (H). Dotted box, center zone of the open field. *n* = 10. I, J, Typical trace (I) and cartogram (J) from the elevated plus maze test. *n* = 10. Among the typical traces (G, I), the CFA, vehicle and exercise group (CFA & Veh & Ex) trace is shown at the top, and the CFA, PCPA and exercise group (CFA & PCPA& Ex) trace is shown at the bottom. K, L, Typical images of MED64 recordings of the fEPSP slope between the CFA, vehicle and exercise group (CFA & Veh & Ex) and the CFA, PCPA and exercise group (CFA & PCPA& Ex). M, Statistical chart of the MED64 recordings of the fEPSP slope during the last 10 min. *n* = 6–7 slice. Unpaired Student's *t* tests were used to compare the CFA, vehicle and exercise group (CFA & Veh & Ex) and the CFA, PCPA and exercise group (CFA & PCPA& Ex) (J, M, **P* < 0.05). Mann-Whitney U test was used to compared the CFA, vehicle and exercise group (CFA & Veh & Ex) and the CFA, PCPA and exercise group (CFA & PCPA& Ex) (H, **P* < 0.05). One-way ANOVA followed by Bonferroni's multiple comparison test was used to compare the Sal & Sed and CFA & Sed groups and the CFA & Sed and CFA & Ex groups (A, ****P* < 0.001, **P* < 0.05). Repeated one-way ANOVA was used to compare the time points (C-F, *****P* < 0.0001, ****P* < 0.001). The data are presented as the mean ± SEM.

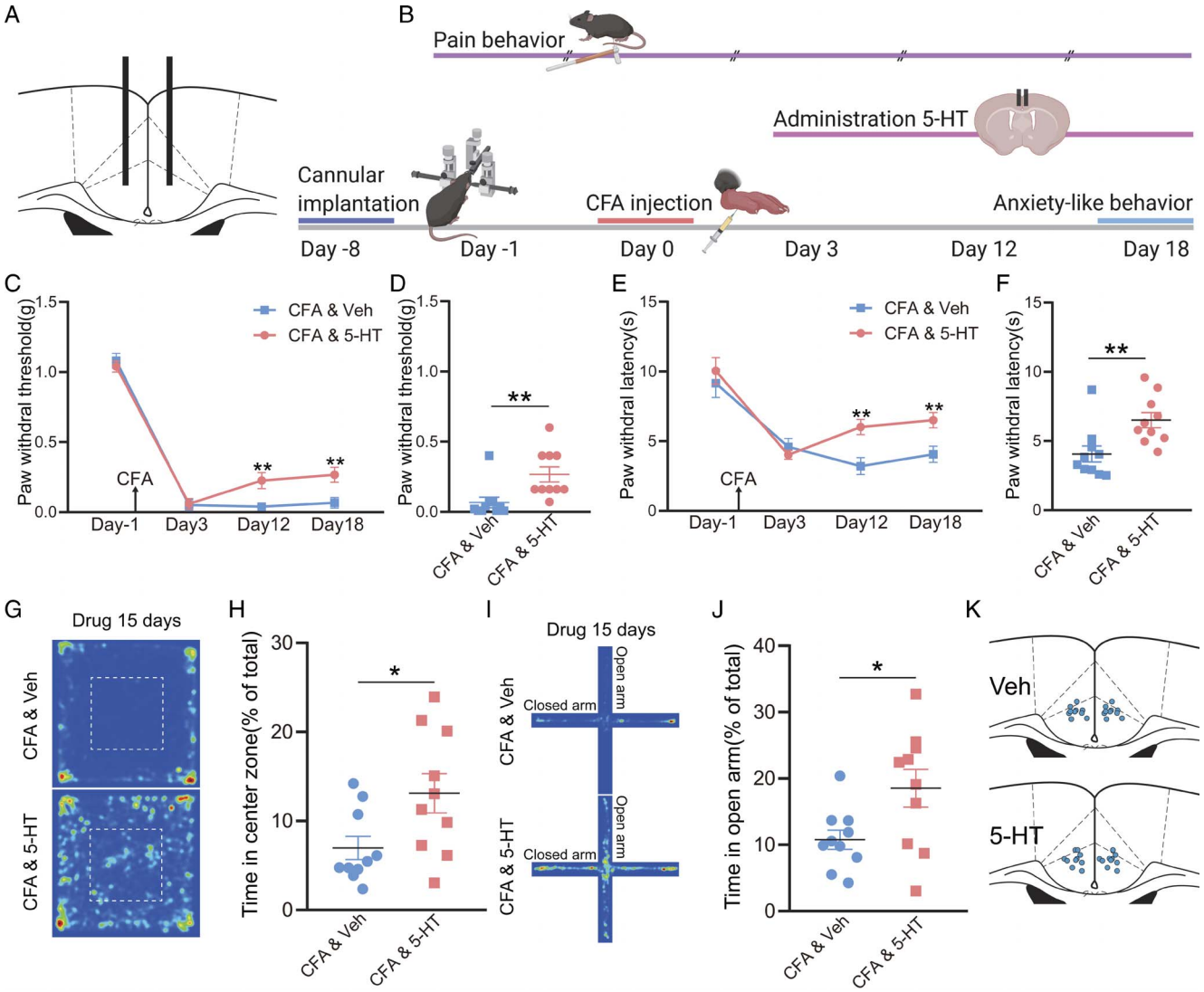

FIGURE 4—Exogenous replenishment of serotonin in the ACC relieves pain and concomitant anxiety. A, Schematic drawing. Cannulation and microinjection into the bilateral ACC. B, Experimental schematic. Cannulas were implanted 7 d before the behavior test. Serotonin (3.5 μg) was administered bilaterally into the ACC through the cannula daily after the pain behavior test starting at postoperative day 3 and continuing until the end of the experiment. C, D, Effect of serotonin administration in the ACC on mechanical allodynia during the time course (C) and at day 18 (D). *n* = 10. E, F, Effect of serotonin administration in the ACC on thermal hyperalgesia during the time course (E) and at day 18 (F). *n* = 10. G, H, Typical trace (G) and cartogram (H) from the open field test. Dotted box, center zone of the open field. *n* = 10. (I, J) Typical trace (I) and cartogram (J) from the elevated plus maze test. *n* = 10. Among the typical traces (G, I), the CFA and vehicle group (CFA & Veh) trace is shown at the top, and the CFA and serotonin group (CFA & 5-HT) trace is shown at the bottom. K, Schematic drawing. The locations of the cannulas in the ACC are shown. Unpaired Student's *t* tests were used to compare the CFA and vehicle (CFA & Veh) and the CFA and serotonin (CFA & 5-HT) groups (H, J, **P* < 0.05) and Repeated One-way ANOVA was used to compare the time points (C–F, ***P* < 0.01). The data are presented as the mean ± SEM.

were conducted using GraphPad Prism 8.0 and SPSS software (v23).

RESULTS

Regular aerobic exercise relieves pain and concomitant anxiety. For the 15-d wheel running exercise, we did not observe significant difference in total distance traveled, duration and running speed between the CFA and saline groups during the 15-d wheel running (Figs. 1B, C, see Figure, Supplemental Digital Content 4, The wheel running speed during the 30-min training period, http://links.lww.com/MSS/C463). The mice could learn to run in the wheel fast and reach to a consistent physical activity in the first day for exercise, which would be maintained throughout the training process (see Figure, Supplemental Digital Content 5, The wheel running speed, distance and duration during the 30-min training period, http://links.lww.com/MSS/C464). Mechanical allodynia and thermal hyperalgesia tests showed that, compared with the CFA sedentary control group, the CFA exercise group exhibited partial rescue of the ipsilateral mechanical threshold and withdrawal latency starting 6 d after exercise began (Figs. 1D–G). In addition, compared with the saline sedentary control mice, the ipsilateral mechanical threshold and withdrawal latency were significantly decreased

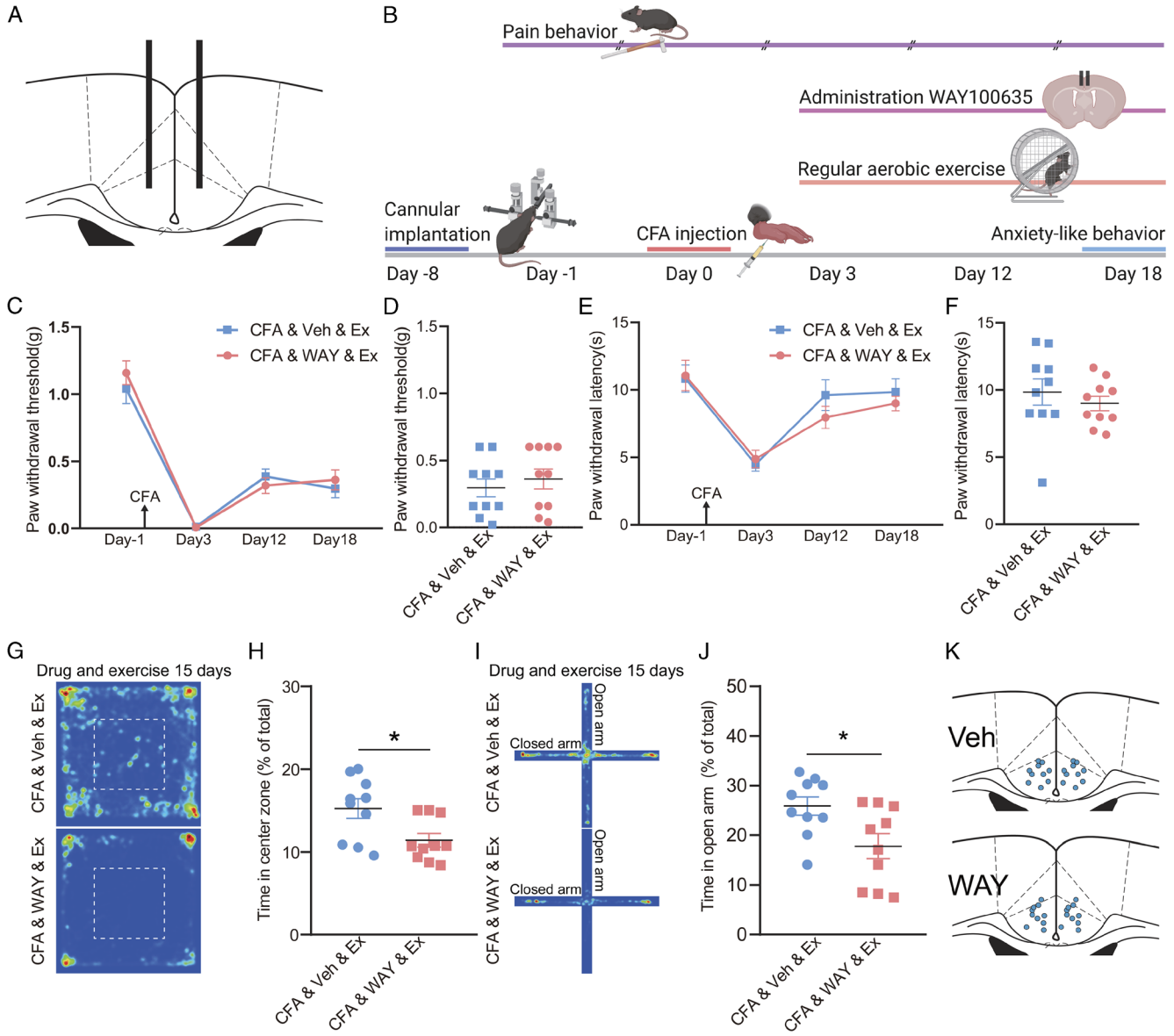

FIGURE 5—Pharmacological inhibition of the 5-HT1A receptor in the ACC blocks the ameliorative effects of exercise on anxiety. A, Schematic drawing. The bilateral ACC was cannulated and microinjected. B, Experimental schematic. Cannulas were implanted 7 d before the behavior test. WAY100635 (1 nM), an antagonist of the 5-HT1A receptor, was administered bilaterally into the ACC through the cannula daily after the pain behavior test starting at postoperative day 3 and continuing until the end of the experiment. Voluntary wheel running was permitted for 30 min daily after drug administration. C, D, Effect of WAY100635 administration in the ACC on exercise-induced alleviation of mechanical allodynia during the time course (C) and at day 18 (D). *n* = 10. E, F, Effect of WAY100635 administration in the ACC on exercise-induced alleviation of thermal hyperalgesia during the time course (E) and at day 18 (F). *n* = 10. G, H, Typical trace (G) and cartogram (H) from the open field test. Dotted box, center zone of the open field. *n* = 10. I, J, Typical trace (I) and cartogram (J) from the elevated plus maze test. *n* = 10. Among the typical traces (G, I), the CFA, vehicle and exercise group (CFA & Veh & Ex) trace is shown at the top. The CFA, WAY100635 and exercise group (CFA & WAY100635 & Ex) trace is shown at the bottom. K, Schematic drawing. The locations of the cannulas in the ACC are shown. Unpaired Student's *t* tests were used to compare the CFA, vehicle and exercise group (CFA & Veh & Ex) and the CFA, WAY100635 and exercise group (CFA & WAY & Ex) (H, J, **P* < 0.05), Mann-Whitney U test was used to compared the CFA, vehicle and exercise group (CFA & Veh & Ex) and the CFA, PCPA and exercise group (CFA & PCPA& Ex) (H, **P* < 0.05) and repeated one-way ANOVA was used to compare the time points (C–F). The data are presented as the mean ± SEM.

after CFA injection (Figs. 1D–G). Complete Freund's adjuvant led to inflammatory pain, and regular aerobic exercise partially relieved allodynia and hyperalgesia. In addition, anxiety behavior was tested by the open field test and elevated plus maze test at the end of the trial, and we observed the percentage of time spent in the center zone and open arm to measure anxiety. The CFA sedentary group mice spent significantly less time in the center zone and open arm than the saline sedentary group mice (Figs. 1H–K). However, the CFA exercise group mice spent more time in the center zone and open arm than the CFA sedentary group mice (Figs. 1H–K). Total distances in the open field and elevated plus maze tests were not affected (see Figure, Supplemental

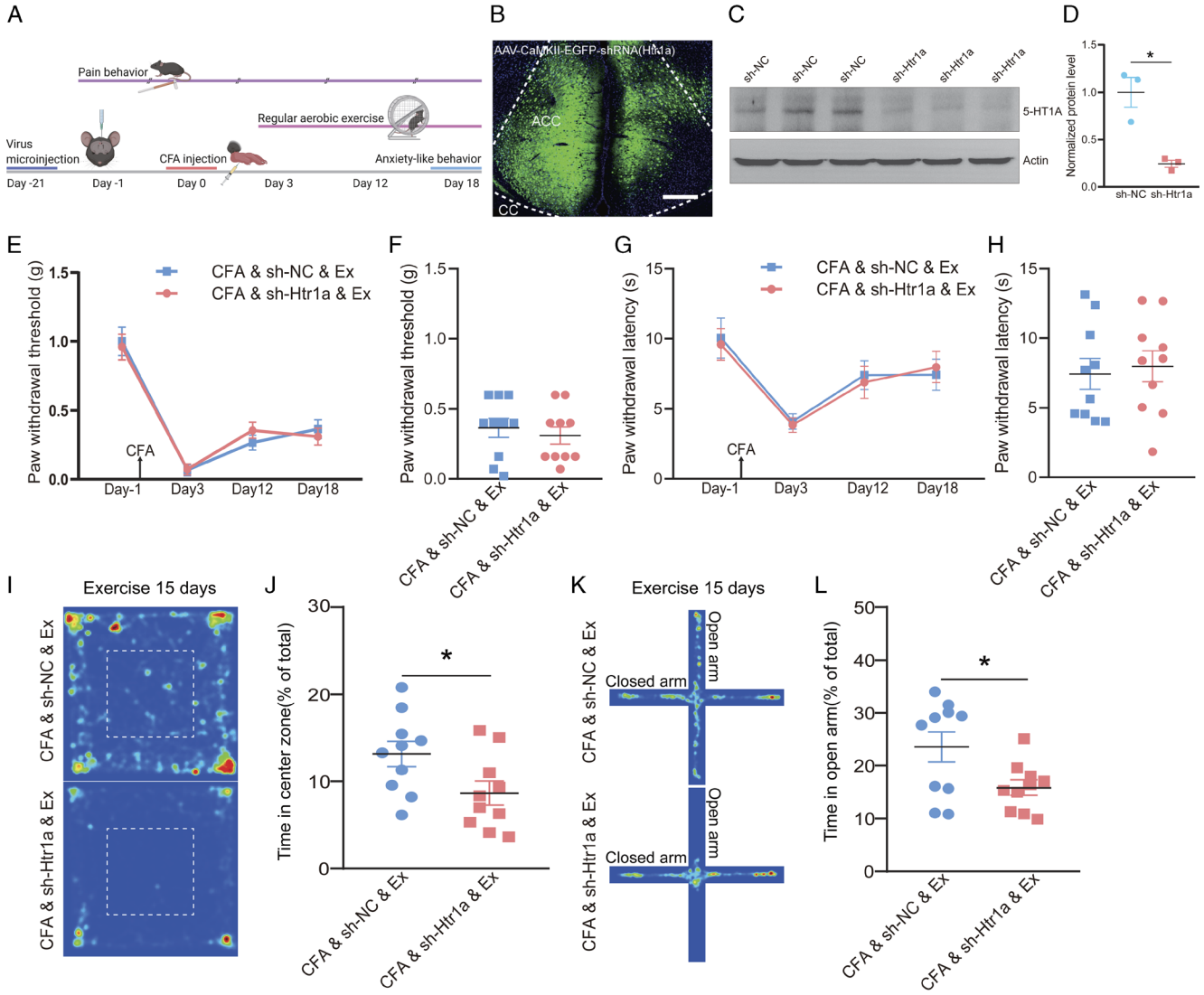

FIGURE 6—Knockdown of the 5-HT1A receptor in the ACC blocks the ameliorative effects of exercise on anxiety. A, Experimental schematic. The virus was injected 21 d before the behavior test. B, Fluorescence image of AAV-CaMKII-EGFP-shRNA (Htr1a) infection in the ACC. Scale bar, 100 µm. C, D, Protein level of 5-HT1A. *n* = 3. E, F, Effect of knockdown of the 5-HT1A receptor in the ACC on exercise-induced alleviation of mechanical allodynia during the time course (E) and at day 18 (F). *n* = 10. G, H, Effect of knockdown of the 5-HT1A receptor in the ACC on exercise-induced alleviation of thermal hyperalgesia during the time course (G) and at day 18 (H). *n* = 10. I, J, Typical trace (I) and cartogram (J) from the open field test. Dotted box, center zone of the open field. *n* = 10. K, L, Typical trace (K) and cartogram (L) from the elevated plus maze test. *n* = 10. Among the typical traces (I, K), the CFA & sh-NC & Ex group trace is shown at the top, and the CFA & sh-Htr1a & Ex group trace is shown at the bottom. Unpaired Student's *t* tests were used to compare the CFA, sh-NC and exercise group (CFA & sh-NC & Ex) and the CFA, sh-Htr1a and exercise group (CFA & sh-Htr1a & Ex) (D, J, L, **P* < 0.05), Mann-Whitney U test was used to compared the CFA, vehicle and exercise group (CFA & Veh & Ex) and the CFA, PCPA and exercise group (CFA & PCPA& Ex) (L, **P* < 0.05), and repeated one-way ANOVA was used to compare the time points (E–H). The data are presented as the mean ± SEM.

Digital Content 6A–B, Total distance traveled in the open field test and elevated plus maze test in all experiments, http://links.lww.com/MSS/C465).

Regular aerobic exercise attenuates pain-induced LTP occlusion in the ACC. We assessed the numbers of *c-Fos*–expressing neurons among the saline sedentary group, CFA sedentary group, saline exercise group, and CFA exercise group (Figs. 2A–D). We found increased *c-Fos*–expressing neurons in the CFA sedentary group, and exercise decreased the numbers of *c-Fos*–expressing neurons in a painful state (Fig. 2E).

We next assessed the possible synaptic mechanisms involved in exercise-mediated relief of pain and concomitant anxiety in the ACC. We recorded LTP using multichannel signals through an MED64 system. Field EPSP were captured while stimulating the deep layers (V/VI) of the ACC (Figs. 2F, G). After 0.5 h of stable baseline recordings, TBS was applied to induce LTP for 1 h. Unlike in saline sedentary mice, TBS failed to induce LTP in the active channels in CFA sedentary mice (Figs. 2H–J). In addition, LTP was partially restored in the active channels in the CFA exercise mice (Figs. 2H–L). Together, these results indicate that regular aerobic exercise is involved

in the regulation of pain-related synaptic plasticity in the ACC, which may contribute to the beneficial effect of exercise on pain-related behavior.

Serotonin release in the ACC contributes to LTP rescue and pain and anxiety relief. We measured the serotonin level in the ACC and found that compared with those in the saline sedentary group of mice, the serotonin levels in the CFA sedentary group of mice were significantly lower in the ACC, and exercise helped recover the serotonin levels in the CFA exercise group of mice (Fig. 3A). Compared with the CFA vehicle group, the CFA PCPA group exhibited persistent mechanical allodynia and thermal hyperalgesia, which diminished the exercise effects (Figs. 3C–F). In addition, the open field and elevated plus maze tests showed that, compared with the CFA vehicle group mice, the CFA PCPA group mice spent less time in the center zone and the open field (Figs. 3G–J). However, the total distances in the open field and elevated plus maze tests were also decreased in the CFA PCPA group (see Figure, Supplemental Digital Content 6C–D, Total distance traveled in the open field test and elevated plus maze test in all experiments, http://links.lww.com/MSS/C465). Together, these data showed that exercise had little effect on relieving pain and concomitant anxiety under serotonin-depleted conditions. Furthermore, we investigated the role of serotonin in ACC LTP. We recorded LTP using multichannel signals in an MED64 system. As shown in Figures 3K–M, PCPA diminished the LTP-rescuing effects of exercise.

Compared with the vehicle control group, the serotonin group exhibited partial rescue of the ipsilateral PWT and paw withdrawal latency during the time course (Figs. 4C–F). In addition, compared with the vehicle control group mice, the serotonin group mice spent more time in the center area of the open field and the open arm of the elevated plus maze (Figs. 4G–J), but the total distance traveled in the open field and elevated plus maze were not affected (see Figure, Supplemental Digital Content 6E–F, Total distance traveled in the open field test and elevated plus maze test in all experiments, http://links.lww.com/MSS/C465). The injection sites were confirmed at the end of the experiments (Fig. 4K). Together, the above data showed that exercise led to increases in serotonin levels in the ACC, which restored LTP induction in CFA mice. Via systemic treatment with PCPA and local infusion of serotonin, we established the causal relationship between exercise-related rescue effects and serotonin alterations in the ACC.

5-HT1A and 5-HT7 receptors in the ACC mediate beneficial exercise effects. Mechanical and thermal pain behavior tests showed that the exercise effects were not significantly affected by WAY100635 during the time course in the WAY100635 group compared with the vehicle control group (Figs. 5C–F). Then, in the open field test and elevated plus maze test, the WAY100635 group mice spent less time in the center area of the open field and in the open arm of the elevated plus maze than the control group mice (Figs. 5G–J), but the total distance in the open field and elevated plus maze did not significantly differ (see Figure, Supplemental Digital Content 6G–H, Total distance traveled in the open field test and elevated plus maze test in all experiments, http://links.lww.com/MSS/C465). The injection sites were confirmed at the end of the experiments (Fig. 5K).

To further confirm the pharmacological discovery, we used recombinant adeno-associated virus shRNA-expressing vectors containing Htr1a shRNA (AAV-CaMKIIa-EGFP-miR30shRNA (Htr1a)-WPRE) to knock down the expression of the 5-HT1A receptor specifically in ACC pyramidal neurons. Nonspecific control shRNA (AAV-CaMKIIa-miR30shRNA (NC)-WPRE) served as the control. According to the above experimental process, 3 wk after virus microinjection, the mice were tested as previously described (Fig. 6A). The injection sites were confirmed at the end of the experiments (Fig. 6B). The knockdown efficiency was tested by Western blot analysis (Figs. 6C–D, see, Figure, Supplemental Digital Content 7, Full blots images for Figure 6C, http://links.lww.com/MSS/C466), and the data showed that the adeno-associated virus successfully knocked down the 5-HT1A receptor. The pain behavior assessment data showed that there was no significant difference during the time course between the Htr1a shRNA group and the NC control group (Figs. 6E–H). However, the mice in the Htr1a knockdown group spent less time in the center area of the open field and in the open arm of the elevated plus maze than the mice in the NC control group (Figs. 6I–L), although the total distance traveled in these two anxiety-like behavior tests was not affected (see Figure, Supplemental Digital Content 6I–J, Total distance traveled in the open field test and elevated plus maze test in all experiments, http://links.lww.com/MSS/C465).

On day 12, the PWT and paw withdrawal latency were significantly reduced in the SB269970 group (Figs. 7C–F). However, the time spent in the center area of the open field and the open arm of the elevated plus maze were not significantly different in the SB269970 group compared with the vehicle control group (Figs. 7G–J). In addition, the total distances traveled in the open field and elevated plus maze were not significantly affected (see Figure, Supplemental Digital Content 6K–L, total distance traveled in the open field test and elevated plus maze test in all experiments, http://links.lww.com/MSS/C465). The injection sites were confirmed at the end of the experiments (Fig. 7K).

Western blot tests were used to assess the knockdown efficiency, and the data showed that the adeno-associated virus successfully knocked down the 5-HT7 receptor (Fig. 8C, D, see Figure, Supplemental Digital Content 8, full blots images for Figure 8C, http://links.lww.com/MSS/C467). The ipsilateral PWT and paw withdrawal latency were decreased at day 18 in the Htr7 knockdown group compared with the NC control group (Figs. 8E–H). In addition, the time spent in the center area of the open field and the open arm of the elevated plus maze were not significantly affected by 5-HT7 receptor knockdown in the ACC (Figs. 8I–L). Furthermore, the total distances traveled in the open field and elevated plus maze were not significantly altered (see Figure, Supplemental Digital Content 6M–N, Total distance traveled in the open field test and elevated plus maze test in all experiments, http://links.lww.com/MSS/C465). These results demonstrated that through pharmacological

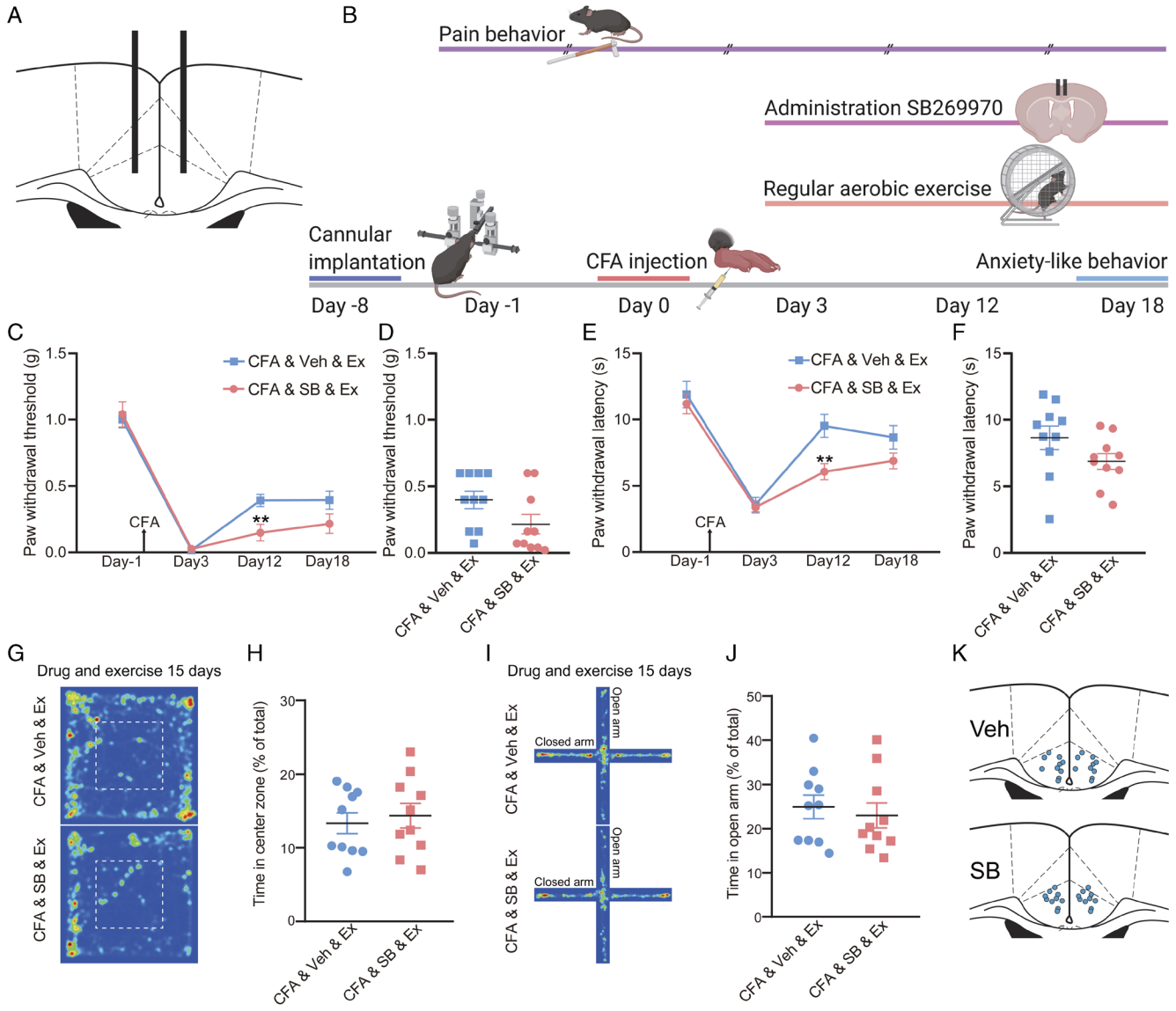

FIGURE 7—Pharmacological inhibition of the 5-HT7 receptor in the ACC blocks the ameliorative effects of exercise on pain. A, Schematic drawing. The bilateral ACC was cannulated and microinjected. B, Experimental schematic. Cannulas were implanted 7 d before the behavior test. SB269970 (100 μM), an antagonist of the 5-HT7 receptor, was administered bilaterally into the ACC through the cannulas daily after the pain behavior test starting on postoperative day 3 and continuing until the end of the experiment. Voluntary wheel running was permitted for 30 min daily after drug administration. C, D, Effect of SB269970 administration in the ACC on exercise-induced alleviation of mechanical allodynia during the time course (C) and at day 18 (D). *n* = 10. E, F, Effect of SB269970 administration in the ACC on exercise-induced alleviation of thermal hyperalgesia during the time course (E) and at day 18 (F). *n* = 10. G, H, Typical trace (G) and cartogram (H) from the open field test. Dotted box, center zone of the open field test. *n* = 10. I, J, Typical trace (I) and cartogram (J) from the elevated plus maze test. *n* = 10. Among the typical traces (G, I), the CFA, vehicle and exercise (CFA & Veh & Ex) trace is shown at the top, and the CFA, SB269970 and exercise group (CFA & SB269970 & Ex) trace is shown at the bottom. K, Schematic drawing. The locations of the cannulas in the ACC are shown. Unpaired Student's *t* tests were used to compare the CFA, vehicle and exercise (CFA & Veh & Ex) and the CFA, SB269970 and exercise (CFA & SB & Ex) groups (H, J), and repeated one-way ANOVA was used to compare each time points (C–F, ***P* < 0.01). The data are presented as the mean ± SEM.

or genetic methods, blocking or knocking down the 5-HT7 receptor had obvious blocking effects on exercise-induced improvements in inflammatory pain.

DISCUSSION

Our study shows, for the first time, that the ACC area is important for regular aerobic exercise-mediated amelioration of inflammatory pain and concomitant anxiety and provides evidence of cellular and molecular mechanisms involved in the regulation of the beneficial effects of exercise. We found that regular aerobic exercise promotes serotonin release and regulates synaptic plasticity in the ACC and that the functions of the 5-HT1A receptor and 5-HT7 receptor in the ACC play important roles in regulating inflammatory pain and concomitant anxiety.

Exercise can improve pain and anxiety. Previous clinical studies have reported that exercise is a feasible, well tolerated,

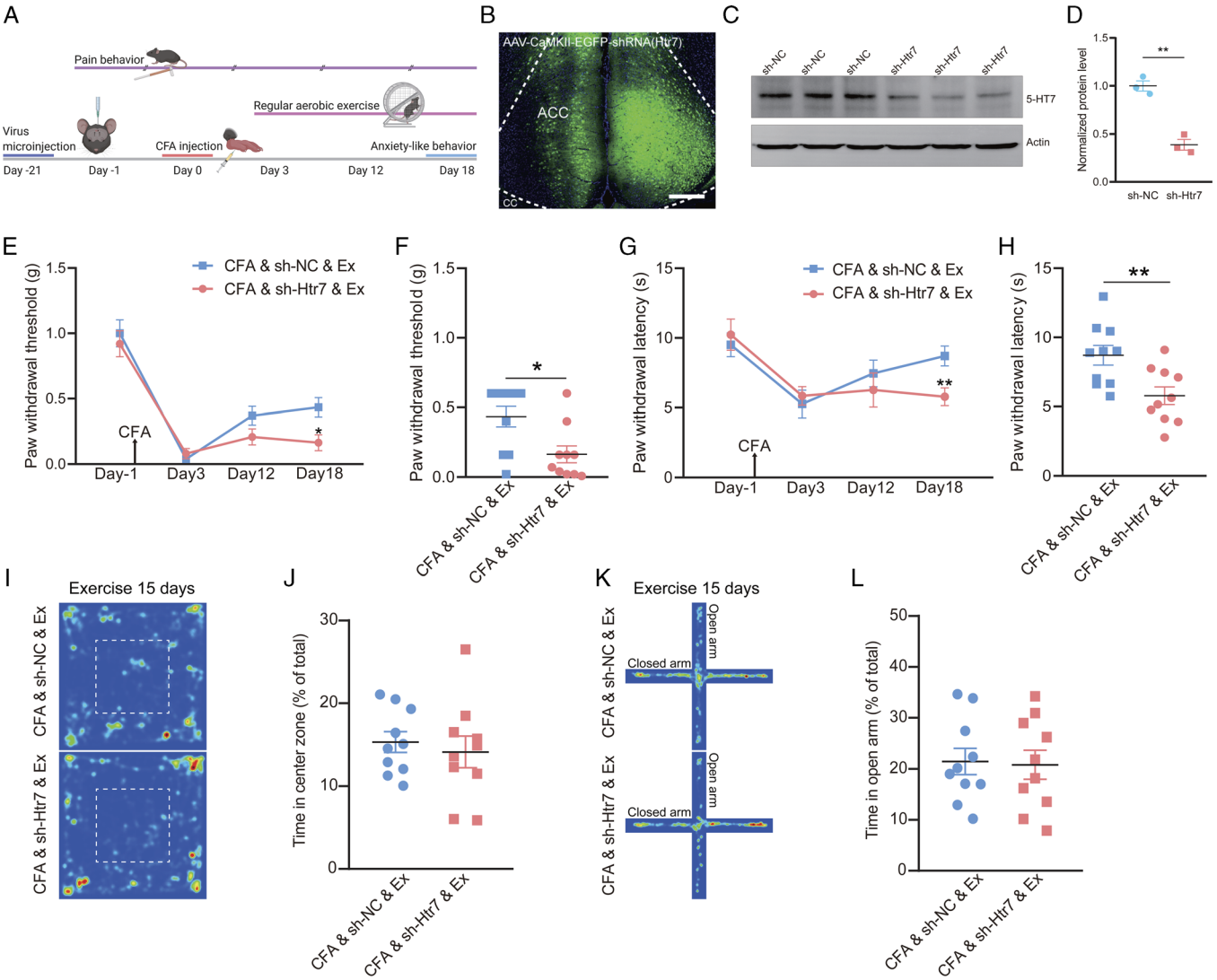

FIGURE 8—Knockdown of the 5-HT7 receptor in the ACC blocks the ameliorative effects of exercise on pain. A, Experimental schematic. The virus was injected 21 d before the behavior test. B, Fluorescence image of AAV-CaMKII-EGFP-shRNA (Htr7) infection in the ACC. Scale bar, 100 μm. C, D, Protein level of the 5-HT7 receptor. *n* = 3. (E, F) Effect of knockdown of the 5-HT7 receptor in the ACC on exercise-induced alleviation of mechanical allodynia during the time course (E) and at day 18 (F). *n* = 10. G, H, Effect of knockdown of the 5-HT7 receptor in the ACC on exercise-induced alleviation of thermal hyperalgesia during the time course (G) and at day 18 (H). *n* = 10. I, J, Typical trace (I) and cartogram (J) from the open field test. Dotted box, center zone of the open field. *n* = 10. K, L, Typical trace (K) and cartogram (L) from the elevated plus maze test. *n* = 10. Among the typical traces (I, K), the CFA, shRNA and exercise (CFA & sh-NC & Ex) group trace is shown at the top, and the CFA, shRNA and exercise group (CFA & sh-Htr7 & Ex) trace is shown at the bottom. Unpaired Student's *t* tests were used to compare the CFA, sh-NC and exercise group (CFA & sh-NC & Ex) and the CFA, sh-Htr7 and exercise group (CFA & sh-Htr7 & Ex) (D, J, L, **$P < 0.01$), and repeated one-way ANOVA was used to compare the time points (E–H, *$P < 0.05$, **$P < 0.01$). The data are presented as the mean ± SEM.

and effective therapy modality for patients with chronic pain or anxiety (31,32). Therefore, understanding the mechanisms underlying exercise-mediated relief of pain and concomitant anxiety is particularly important and will support the combined application of drug and nondrug therapies in clinical practice. Moreover, a neuroimaging study has reported that exercise increases gray matter volume in the ACC (33). This indicates that the beneficial effects of exercise may be related to the ACC. Consistent with this finding, we found that regular aerobic exercise relieved pain and concomitant anxiety by regulating serotonin release in the ACC and acting on the 5-HT1A receptor and the 5-HT7 receptor. Ultimately, increased serotonin in the ACC contributes to rescue of LTP occlusion and contributes to relieving pain and related anxiety.

Previous animal research has suggested that several modes of exercise, including swimming, treadmill exercise, and wheel running, can improve pain (18,19,34). Moderate treadmill training and voluntary wheel running can both improve pain and anxiety (35). Upon comparing different exercise methods, we have found that the majority of the exercise protocols, regardless of their intensity, improve pain behaviors, but only moderate exercise, such as voluntary wheel running, can improve affective emotions such as anxiety and depression (7). Therefore, the effects of exercise on pain and anxiety behaviors may

depend on the length and intensity of exercise. Consistent with this idea, we found that 30 min of voluntary wheel running attenuated pain and concomitant anxiety. This result suggests that moderate exercise might be appropriate for pain and affective emotion management. Meanwhile, previous study suggested that CFA injected bilaterally in the hindpaws would show a marked decrease in distance traveled (36). However, the authors also suggested that mice injected unilaterally with CFA showed a modest, nonsignificant decrease in distance traveled. Moreover, mice distance traveled recovered to a large extent at 3 d after bilateral hindpaws CFA injection. In line with our study, we started wheel running at 3 d after unilaterally injected CFA. It is implied that inflammation in one hindlimb can be compensated with the other three limbs. Furthermore, our data shown that there was no difference in the total distance traveled between the two groups of mice (Figs. 1B, C, see Figure, Supplemental Digital Content 4, The wheel running speed during the 30-min training period, http://links.lww.com/MSS/C463; and see Figure, Supplemental Digital Content 5, The wheel running speed, distance and duration during the 30-min training period, http://links.lww.com/MSS/C464). In addition, we only used male mice for our experiments. Therefore, whether there is any sex differences related to analgesic and anxiolytic effect of exercise still need to be explored in the future study.

Several studies have shown that LTP is closely related to exercise. Synaptic plasticity, including that associated with long-term potentiation or depression, is an experience-dependent process that results in long-lasting changes in synaptic communication (37). Abnormalities in synaptic function play key roles in several pathological conditions, including chronic pain (38). In this context, a number of studies have focused on therapeutic interventions capable of inducing the molecular and structural changes that lead to increased synaptic efficacy (39). Previous research has found that running enhances LTP in the dentate gyrus of the hippocampus and promotes the cognitive function of rodents (40). In addition, regular exercise prevents sleep deprivation-induced LTP deficits in the dentate gyrus of the hippocampus, indicating protective effects against impairments caused by sleep deprivation (41). Consistent with this finding, we found that regular aerobic exercise promoted the rescue of LTP occlusion induced by pain in the ACC. When we systemically delivered PCPA, an endogenous drug for depletion of central serotonin, we found that the ameliorative effect of exercise on LTP disappeared, and the analgesic and antianxiety effects of exercise were also blocked. Our results reveal the necessary role of exercise-induced serotonin release in the beneficial effects of exercise and suggest that the ACC plays a critical role in the ameliorative effects of regular aerobic exercise on chronic pain.

Serotonin is one of the most studied neurotransmitters related to chronic pain. Cumulative evidence from clinical research shows that serotonin (5-HT) reuptake inhibitors are effective in treating pain and pain-related syndromes (42,43). A previous study has pointed out that local administration of serotonin in the PFC has antianxiety effects but does not have analgesic effects. However, other animal studies have shown that increased serotonin in certain brain areas involving the ventrolateral orbital cortex is related to reduced pain and pain-related behavior (8,9). Through long-term chronic administration of serotonin in our experiments, we found that serotonin has analgesic effects with increasing administration period. On the one hand, this set of experiments suggests that there may be different roles between the different brain areas. On the other hand, the findings might also suggest that it takes a certain amount of time to reach an appropriate drug concentration for serotonin to exert analgesic effects. 5-HT is a key neurotransmitter that suppresses the transmission of painful peripheral stimuli by inhibiting input to spinal dorsal horn neurons (44). Moreover, the dorsal raphe nucleus is known to send descending serotoninergic projections to the spinal cord (45). Previous studies using different methods have reported that one role of serotonin in the spinal cord is to alleviate pain (46). However, for modulation of pain-related affective behaviors, the ACC could be one of the critical hubs in the brain. The ACC receives a variety of sensory information inputs, and the synaptic transmission efficiency of the ACC is increased under pain conditions, leading to excessive amplification of sensory information, which promotes the transmission of pain information in the descending pain facilitation pathway. With exercise training, serotonin may improve the neural plasticity of ACC pyramidal neurons, which might restore the function of the pain facilitation pathway. However, how serotonin modulates the circuits and the mechanisms underlying the synaptic alterations still need further exploration. In addition, a small amount of adjacent brain regions might be collected when we studied the ACC, since it would be very difficult to collect ACC tissue only, even we have paid close attention while tissue dissecting. Whether the adjacent brain regions would also show the similar alterations still need to be explored in the future.

In the ACC, there are many subtypes of serotonin receptors. According to previous reports, 5-HT1A and 5-HT7 receptors play important roles in pain conditions (15,30,47). Therefore, to further explore ACC downstream targets for increased release of serotonin induced by exercise, we conducted further studies on 5-HT1A and 5-HT7 receptors. Consistent with the other results, we found that the analgesic and antianxiety effects of exercise intervention were changed after 5-HT1A and 5-HT-7 receptor antagonists were delivered by cannula into the ACC. After administration of a 5-HT1A receptor antagonist, we found that exercise still improved pain, but the improvement in anxiety was partially blocked. However, after administration of the 5-HT7 receptor antagonist, the analgesic effect of exercise was partially blocked on the 12th day but gradually recovered by the 18th day, and the 5-HT7 receptor antagonist had little blocking effect on the antianxiety effect. Furthermore, we knocked down the two receptors through shRNA. In line with the pharmacological results, the ameliorative effects of exercise on pain still existed, but the improvement of anxiety was partially blocked when we knocked down the 5-HT1A receptor in the ACC. However, after knockdown of the 5-HT7 receptor, the analgesic effect of exercise was partially blocked on the 18th day but was not blocked on the 12th

day, and knockdown had little blocking effect on the antianxiety effect. The above results suggest that serotonin is likely to mediate the analgesic and antianxiety effects of exercise through two different receptors. However, the difference in PWT and latency between the 12th and 18th days after blockade or knockdown of the 5-HT7 receptor was probably due to the differences in the experimental methods. First, pharmacological experiments inhibit the functioning of receptors through receptor antagonists, whereas genetic experiments decrease the functioning of receptors via knockdown of the expression of the receptors. The different strategies might lead to different amounts of functional receptors in different experimental stages. Second, pharmacological experiments inhibit the receptor after pain is established, whereas genetic experiments reduce the expression of the receptor before pain is established. Although the pharmacological and genetic manipulation of 5-HT1A and 5-HT7 receptors resulted in some inconsistency in the behavioral tests, these results still demonstrate that 5-HT1A and 5-HT7 receptors are involved in the beneficial effects of exercise on pain and anxiety. Furthermore, our results show that the 5-HT1A receptor mainly affects the sensory component of pain, whereas the 5-HT7 receptor mainly affects the affective component of pain. A previous report has shown that the ACC might preferentially modulate anxiety and chronic pain through presynaptic and postsynaptic mechanisms (38). According to other researches, 5-HT1A receptors mainly exert their effects by influencing the release of presynaptic neurotransmitters (15), whereas 5-HT7 receptors mainly affect the function of dendritic integration, thus targeting a postsynaptic mechanism (48). Consistent with this idea, we found that 5-HT1A and 5-HT7 receptors might contribute to exercise-induced pain and anxiety improvement in different manners. Since there are multiple subtypes of serotonin receptors, whether different subtypes have different effects in the ACC region needs further research. In addition, in the future, the three-dimensional crystal structures of serotonin receptors can be analyzed, providing the possibility for further exploration of new drug treatment targets.

CONCLUSIONS

In the present work, we used several approaches, including electrophysiological, pharmacological, biochemical, behavioral, and genetic approaches, to determine whether a regimen of regular aerobic exercise could relieve pain and concomitant anxiety and to elucidate the possible mechanisms underlying the analgesic and antianxiety effects of exercise. Overall, our study demonstrates that regular aerobic exercise can relieve pain and anxiety. An increase in serotonin in the ACC contributes to rescue of LTP occlusion, and the 5-HT1A receptor and the 5-HT7 receptor are downstream targets that exert the beneficial effects of exercise.

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