The Impact of Electroacupuncture Early Intervention on the Brain Lipidome in a Mouse Model of Post-traumatic Stress Disorder

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Abstract

The neuroprotective effect of electroacupuncture (EA) treatment has been well studied; growing evidence suggests that changes in lipid composition may be involved in the pathogenesis of posttraumatic stress disorder (PTSD) and may be a target for treatment. However, the influence of early EA intervention on brain lipid composition in patients with PTSD has never been investigated. Using a modified single prolonged stress (mSPS) model in mice, we assessed the anti-PTSD-like effects of early intervention using EA and evaluated changes in lipid composition in the hippocampus and prefrontal cortex (PFC) using a mass spectrometry-based lipidomic approach. mSPS induced changes in lipid composition in the hippocampus, notably in the content of sphingolipids, glycerolipids, and fatty acyls. These lipid changes were more robust than those observed in the PFC. Early intervention with EA after mSPS ameliorated PTSD-like behaviors and partly normalized mSPS-induced lipid changes, notably in the hippocampus. Cumulatively, our data suggest that EA may reverse mSPS-induced PTSD-like behaviors due to region-specific regulation of the brain lipidome, providing new insights into the therapeutic mechanism of EA.

Introduction

Posttraumatic stress disorder (PTSD) is a stressor-related disorder characterized by avoidance, re-experiencing of trauma, and hyperarousal symptoms, and affects approximately 7–10% of the general population [1–3]. It is worth noting that the prevalence of PTSD increased significantly after the start of the COVID-19 pandemic [4, 5]. PTSD is often associated with other psychiatric symptoms and functional impairment. The disorder may persist over the patient's lifetime and entail costs higher than those associated with other anxiety disorders [6–8]. Currently, the main treatment strategy for PTSD is psychological intervention combined with pharmacotherapy. However, psychological intervention provides only partial benefits [9]. Moreover, the benefits of drug treatments decline over time and drug use carries a risk of withdrawal syndromes and relapse [10]. Investigation of the pathogenesis of PTSD and the development of new approaches for treating it are thus urgently needed [11, 12].

Electroacupuncture (EA) is a modified form of traditional acupuncture. The latter is a technique of traditional Chinese medicine typically used in the field of complementary and alternative medicine [13]. Previous studies have mainly focused on the analgesic effect of EA [14]. However, multiple studies have found that electroacupuncture not only has neuromodulatory effects [15, 16], but can also ameliorate the symptoms of several neuropsychiatric diseases [17–19]. Interestingly, a large number of studies have reported that EA exerts neuroprotective effects by regulating the structure and function of the hippocampus and prefrontal cortex (PFC) [20–22], which are regions of central importance in PTSD due to their prominent role in the neuroendocrine stress response and memory [23–25]. Clinical and preclinical studies have previously reported that EA is protective against PTSD [26, 27], and our previous work further found that early intervention or pretreatment using EA could prevent PTSD-like behaviors in a rat model of PTSD [28, 29]. Taken together, these results show that EA treatment is potentially therapeutic in cases of PTSD.

Lipids play an important role in maintaining and regulating brain development and function. Such functions include learning/memory and emotional behavior [30–32]. The involvement of lipids in modulating synaptic physiology, receptor pharmacology, energy generation, and brain metabolism has largely been established [33–35]. Recently, lipidomic alterations have been reported in psychiatric disorders, including bipolar disorder [36, 37], schizophrenia [38], and anxiety disorder [39]. Peripheral lipids may also be promising biomarker candidates to assist in the differential diagnosis of mild traumatic brain injury and PTSD [40]. However, to our knowledge, the changes in the brain lipidome occurring in PTSD and the link between the neuroprotective effects of EA and lipidomic alterations remain elusive.

Considering the above, we aimed to determine the influence of early EA intervention on the lipidomic composition of the hippocampus and PFC in a mouse model of modified, single prolonged stress (mSPS), which can produce robust symptoms and enhanced conditioned and sensitized fear responses that mimic PTSD [41]. We also aimed to determine the lipid changes characteristic of PTSD and EA treatment.

Materials And Methods

Animals

Adult male C57BL/6 mice (8 weeks old, weighing 18–22 g) were obtained from the Fourth Military Medical University Animal Center (Xi'an, China). The mice were group-housed at 4 per cage in wire-bottomed cages at 20–25°C and maintained on a 12 h light/12 h dark daily cycle (lights on from 8:00 AM to 8:00 PM). Food and water were available ad libitum and all experiments were conducted during the light phase. The experimental procedures of this study were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Use and Protection Committee of the Fourth Military Medical University.

Experimental design

As shown in Figure 1A, after seven days of acclimatization, 32 mice were randomly assigned to the following four groups (n = 8/group): Sham, EA, PTSD, and PTSD+EA. Mice in the PTSD and PTSD+EA groups were treated with mSPS. Subsequently, mice in the EA and PTSD+EA groups were stimulated by EA at a frequency of 2/15 Hz and an intensity of 1 mA for 30 min each day, for 7 consecutive days. Mice in the Sham and PTSD groups were administered false stimulation (EA treatment without application of electric current) for 30 min each day for 7 consecutive days, and then handled in the home cage for one week. The researchers performing the behavioral testing were blinded to the animals' group allocations, and the behavioral tests were administered one week after the final intervention. Subsequently, the mice were sacrificed and the PFC and hippocampus were immediately collected in liquid nitrogen for later lipidomic analyses by high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS).

mSPS (modified single prolonged stress)

The modified SPS paradigm was as described in a previous study [42], with some modifications. Briefly, mice were restrained for 2 h and then immediately administered forced swimming for 20 min in an acrylic cylindrical tank (10 cm diameter, 25 cm height, water temperature 20–24°C) filled approximately two-thirds with water. Following gentle drying, the mice were subsequently exposed to diethyl ether until they lost consciousness for 2–3 min. After a 15-min recovery, the mice were exposed to a single electric foot shock (0.75 mA for 2 s) in a square box (360 mm width × 360 mm height × 360 mm depth) with a floor of stainless-steel rods and walls of aluminum and acrylic. Subsequently, the mice were returned to their home cages.

EA treatment

The electroacupuncture treatment method was as described previously [43]. Mice were anesthetized with isoflurane (1.5 × minimum alveolar concentration) during EA stimulation and the “Bai hui” acupoint (GV20), which is located at the intersection of the sagittal midline and the line linking the ears, was stimulated for 30 min daily at a frequency of 2/15 Hz and an intensity of 1 mA (waveform: dilatational wave) with a G6805–2 electric acupuncture apparatus (No. 227033; Qingdao Xinsheng Ltd.). False stimulation was performed at the same acupoint without electricity (Mice receiving false stimulation were also anesthetized with isoflurane).

Behavioral testing

Behavioral testing occurred 14 days after mSPS (see Figure 1A). Animals were acclimated to a separate experimental room for at least 30 min prior to each test. The open field test was conducted prior to the elevated plus maze test on the same day, while the fear conditioning test was performed 24 h after the elevated plus maze test. All experiments were conducted under low light conditions to minimize anxiety effects and the test area was cleaned with 75% ethanol between tests.

Open-field test (OFT)

Based on a previous study [44], the OFT was performed in an unfamiliar soundproof box (50 × 50 × 50 cm) made of white polycarbonate. The floor had black grid marks dividing the behavioral arena into 36 squares comprising 16 central and 20 peripheral squares. Animals were placed in the center of the open-field box and activity was recorded for a period of 5 min by a video camera positioned directly above the arena. The recordings were analyzed by the open-field activity software Top Scan (Clever Sys Inc., USA). The time spent and distance travelled in the central squares and entire arena were measured.

Elevated-plus maze test (EPMT)

The EPMT was used to detecting anxiety-like behavior in mice [45]. The maze apparatus (Dig Behav, Ji liang Co. Ltd., Shanghai, China) consisted of two opposing open arms of 35 × 6 cm and two opposing enclosed arms of 35 × 6 cm, all elevated 50 cm above the floor. During a test, a mouse was placed in the center square of the maze facing an open arm and its behavior recorded for 5 min by an automatic

analyzing system (Top Scan, Clever Sys Inc., USA). The number of arm entries and the time spent in open arms were used as indices of anxiety.

Fear conditioning response (FCR) test

Conditioned fear behavior was examined by the FCR test as described in previous reports [42]. The experiments were performed in two dissimilar chambers: a shock chamber (Context A: a rectangular box with floors made of stainless-steel rods and walls of aluminum and acrylic) and a neutral-context chamber (Context B: a rectangular box with a white acrylic floor and an acrylic frame roof). The contextual fear conditioning paradigm and the cued fear conditioning paradigm each consisted of one training and two test sessions. For training, mice were acclimated to the shock chamber (Context A) for 180 s, then presented with a pure tone (28 s, 1 kHz, 80 dB) immediately followed by a foot shock (2 s, 0.75 mA). The tone–foot-shock pairing was delivered twice followed by a 90-s rest period, also in the chamber. Twenty-four hours later, mice were placed in the contextual chamber (Context A) for 5 min, without exposure to tone or foot shock, for the contextual fear response test. The cued fear response test was performed 1 h later. In this test, mice were acclimated to the neutral chamber (Context B) for 3 min. Subsequently, a neutral tone (4 kHz, 80 dB) was presented without foot-shock for 3 min. Mice were returned to the home cage after resting in the neutral chamber (Context B) for another 60 s. Freezing behavior was recorded and analyzed using a computerized automatic analysis system (Freezing Scan, Clever Sys Inc., Reston, VA, USA).

Tissue collection

Mice were euthanized by cervical dislocation within 4 h of the end of behavioral testing. The brains were subsequently removed and the PFC and hippocampi were dissected on ice, then precisely weighed, frozen in liquid nitrogen, and stored until use.

Sample extraction

Lipids were extracted with methyl tert-butyl ether (MTBE) as described in a previous study [46]. Briefly, 30 mg samples were spiked with appropriate amounts of internal lipid standards (20 μL) and then homogenized with 200 μL of water and 240 μL of methanol. Subsequently, 800 μL of MTBE were added, and samples were ultrasonicated for 20 min at 4°C, then incubated at 25°C for 30 min. To separate the organic components, the solution was centrifuged at 10°C for 15 min at 14,000 × *g*. The upper layer, which was the organic solvent phase, was collected and the solvent evaporated under nitrogen. The solutes were stored at -80°C until use.

Lipid analysis by HPLC–MS/MS

Reverse-phase chromatography was selected for ultra-HPLC separation using a CSH C18 column (1.7 μm, 2.1 × 100 mm, Waters). The lipid extracts were re-dissolved in 200 μL of 90% isopropanol/acetonitrile and centrifuged at 14,000 × *g* for 15 min; finally, 3 μL of each sample was injected for analysis. Solvent A was acetonitrile–water (6:4, v/v) containing 0.1% formic acid and 0.1 mM ammonium formate; solvent B was

acetonitrile–isopropanol (1:9, v/v) also containing 0.1% formic acid and 0.1 mM ammonium formate. The initial mobile phase was 30% solvent B at a flow rate of 300 μL/min, which was held for two min, then linearly increased to 100% solvent B over 23 min, followed by equilibration in 5% solvent B for 10 min. The autosampler was maintained at 10°C. To avoid bias due to instrumentation errors, multiple samples were analyzed concurrently and signal fluctuations were detected in random order, even during sample analysis. Sampling of the queue was performed every eight samples using one of the quality control (QC) samples to monitor the stability of the analysis and evaluate the reliability of the experimental data.

After separation by ultra-HPLC, samples were analyzed using a Q Exactive™ plus mass spectrometer (Thermo Fisher Scientific, Waltham, USA) using the following parameters. For positive-ion mode: heater temperature, 300°C; sheath gas flow rate, 45 arbitrary units (arb); auxiliary gas flow rate, 15 arb; sweep gas flow rate, 1 arb; spray voltage, 3.0 kV; capillary temperature, 350°C; S-Lens radio frequency (RF) level, 50%; and MS1 scan range, 200–1800 m/z. For negative-ion mode: heater temperature, 300°C; sheath gas flow rate, 45 arb; auxiliary gas flow rate, 15 arb; sweep gas flow rate, 1 arb; spray voltage, 2.5 kV; capillary temperature, 350°C; S-Lens RF level, 60%; and MS2 scan range, 250-1800 m/z.

Lipid identification using LipidSearch™

LipidSearch (Thermo Fisher Scientific, Walthamcity, country, USA) is a search engine used to identify lipid species based on MS/MS data. LipidSearch contains data on >30 lipid classes and >1,500,000 ion fragments. The mass tolerances for both molecular precursors and fragment ions were set to 5 ppm. The displayed product ion threshold was set at five, and grades A, B, C, D were all used in the identification (ID) quality filtering. All lipid classes in the database, including 71 sub-species, were chosen for identification. Adducts of H^+ and NH_4^+ were selected for positive-mode searches, and adducts of H^- and CH_3COO^- were selected for negative-mode searches, since ammonium acetate was used in the mobile phases.

Statistical analysis

Statistical analyses were performed using SPSS v.19.0 software (SPSS Inc., Chicago, IL, USA). The results of behavioral testing and the characterizations of lipid compositions are presented as mean ± standard deviation (SD), and were evaluated by one- or two-way analysis of variance (ANOVA) followed by a Bonferroni *post-hoc* test for pairwise comparisons. A *P*-value <0.05 was deemed statistically significant. The correlations between lipid species levels and behaviors were analyzed using the Pearson correlation.

The raw lipidomic data were processed using LipidSearch software for peak recognition, lipid-peak extraction (secondary appraisal), peak alignment, and quantitative processing. After normalization and integration using the Perato scaling method, the processed data were imported into SIMPCA-P 14.1 (Umetrics, Umea, Sweden) for multivariate statistical analysis, which included principal component

analysis, partial least squares discriminant analysis, and orthogonal partial least squares discriminant analysis. Lipids with significant differences were identified based on a combination of 1) statistically significant thresholds of variable influence on projection values (obtained from orthogonal partial least squares discriminant analysis and two-tailed Student's *t*-tests) and 2) mapping of volcano, hierarchical-cluster, and correlation analyses using R software [47].

Results

EA treatment ameliorates anxiety-like behaviors and fear learning defects in mSPS-treated mice

First, we determined the effect of EA treatment on PTSD-like behaviors (Figure 1A). Two-way ANOVA revealed that mSPS and EA treatment did not induce any motor impairment in mice because in the OFT, we observed no significant differences in the total distance traveled in either the mSPS or the EA treatment factors ($F = 2.320$, $P = 0.139$; and $F = 0.270$, $P = 0.871$; respectively; Figure 1B, C). However, we observed significant differences in the time spent in the center squares in the OFT for the mSPS factor ($F = 4.348$, $P = 0.046$, Figure 1D), as well as in the open arms of the EPMT (Figures 1E, F) for both the mSPS ($F = 10.401$, $P < 0.01$) and EA factors ($F = 7.373$, $P = 0.011$). In addition, for the mSPS factor, all differences in freezing time were significant for both contextual fear ($F = 10.587$, $P < 0.01$, Figure 1G) and cued fear ($F = 18.389$, $P < 0.01$, Figure 1H). *Post hoc* comparisons further showed that mSPS markedly reduced the time spent in the center in the OFT and the time spent in the open arms in the EPMT (PTSD *vs.* Sham, $P < 0.05$), and that these measures were ameliorated after EA treatment (PTSD+EA vs. PTSD, $P < 0.05$). Additionally, the PTSD group showed a significant increase in freezing time in both contextual fear and cued fear conditioning tests when compared with the Sham group ($P < 0.01$). Moreover, EA treatment significantly decreased freezing time and enhanced fear learning in the PTSD-treated mice (PTSD+EA *vs.* PTSD, $P < 0.05$, Figure 1G, H). These results suggest that EA treatment can ameliorate anxiety-like behaviors and fear learning defects in PTSD model mice.

EA treatment effect: hippocampal lipid classes

As shown in Figure 2, we found no statistical intergroup differences in total lipid levels ($F = 1.598$, $P = 0.2122$) in the hippocampus (Figure 2A). However, in this structure, we observed significant differences for the mSPS factor in the concentrations of the following: diglyceride (DG; $F = 44.152$, $P < 0.001$), triglyceride (TG; $F = 6.226$, $P = 0.019$), sphingomyelin (SM; $F = 20.162$, $P < 0.001$), ceramides (Cer; $F = 5.807$, $P = 0.023$), gangliosides (GM1; $F = 9.702$, $P = 0.004$), cardiolipin (CL; $F = 28.410$, $P < 0.001$), lysophosphatidylglycerol (LPG; $F = 13.624$, $P = 0.001$), lysophosphatidylinositol (LPI; $F = 11.034$, $P < 0.01$), lysophosphatidylserine (LPS; $F = 14.791$, $P = 0.001$), phosphatidic acid (PA; $F = 12.734$, $P = 0.001$), phosphatidylethanolamine (PE; $F = 37.813$, $P < 0.001$), phosphatidylglycerol (PG; $F = 37.426$, $P < 0.001$), phosphatidylinositol (PI; $F = 16.968$, $P < 0.001$), acyl carnitines (AcCa; $F = 23.807$, $P < 0.001$), sulfoquinovosyldiacylglycerol (SQDG; $F = 21.967$, $P < 0.001$), monogalactosyldiacylglycerol (MGDG; $F = 47.718$, $P < 0.001$), and coenzyme (Co; $F = 45.690$, $P < 0.001$). In addition, we observed significant differences for the EA treatment factor in the concentrations of the following: TG ($F = 60.011$, $P < 0.001$), GM1 ($F = 12.357$, $P = 0.002$), SM ($F = 43.338$, $P < 0.001$), Cer ($F = 14.462$, $P < 0.01$), sphingosine (So; $F =$

15.388, $P = 0.001$), CL ($F = 44.882$, $P < 0.001$), lysophosphatidylethanolamine (LPE; $F = 5.653$, $P = 0.024$), LPG ($F = 13.004$, $P = 0.001$), LPS ($F = 30.514$, $P < 0.001$), PA ($F = 4.757$, $P = 0.038$), PE ($F = 19.787$, $P < 0.001$), PG ($F = 19.259$, $P < 0.001$), AcCa ($F = 18.013$, $P < 0.001$), fatty acid (FA; $F = 34.261$, $P < 0.001$), digalactosyldiacylglycerol (DGDG; $F = 27.467$, $P < 0.001$), SQDG ($F = 43.754$, $P < 0.001$), MGDG ($F = 95.459$, $P < 0.001$), and Co ($F = 22.278$, $P < 0.001$). *Post hoc* comparisons revealed that mice in the PTSD group exhibited significantly elevated levels of DG, TG, Cer, GM1, LPS, lysophosphatidylcholine (LPC), PI, and PE but reduced levels of SM, CL, LPI, FA, AcCa, and Co (PTSD *vs.* Sham, $P < 0.05$). EA treatment effectively deceased the levels of Cer, GM1, LPS, PE, and TG and increased the levels of SM, CL, AcCa, FA, and Co (PTSD+EA *vs.* PTSD, $P < 0.05$). However, the changes in DG, LPI, LPC, and PI induced by mSPS in the hippocampus were not normalized by EA treatment.

Correlation analysis showed that the time spent in the center squares in the OFT was positively correlated with levels of AcCa, CL, LPI, SM, Co, and DGDG but negatively correlated with levels of TG, LPS, and PE. The time spent in the open arms in the EPMT was positively correlated with levels of FA, CL, LPG, LPI, PC, SM, Co, and DGDG but negatively correlated with levels of DG, TG, LPC, LPS, PE, Cer, and GM1. Moreover, the contextual freezing time recorded in the FCR test was positively correlated with levels of DG, LPC, LPS, PE, and PI but negatively correlated with levels of AcCa, FA, CL, LPG, LPI, SM, and Co. The cued FCR freezing time was positively correlated with levels of DG, LPC, LPS, PE, PI, and Cer but negatively correlated with levels of AcCa, FA, CL, LPI, and Co (Figure 4A and Table S1). These results suggest that hippocampal levels of AcCa, FA, CL, LPI, SM, and Co are negatively correlated with the severity of PTSD, while levels of DG, LPC, PE, LPS, and Cer are positively correlated with severity.

EA treatment effect: PFC lipid classes

As shown in Figure 3, we observed significant intergroup differences in total lipid levels in the PFC ($F_{3,28} = 39.91$, $P < 0.001$, Figure 3A); mSPS treatment decreased the total lipid concentration in this region (PTSD *vs.* Sham, $P < 0.01$). Moreover, we found significant differences for the mSPS factor in the levels of the following lipids in the PFC: FA ($F = 23.768$, $P < 0.001$), wax esters (WE; $F = 16.173$, $P < 0.001$), Cer ($F = 16.882$, $P < 0.001$), simple Glc series (CerG2GNAc1; $F = 6.514$, $P = 0.016$), GM1 ($F = 12.544$, $P = 0.001$), sphingomyelin (phSM; $F = 15.960$, $P < 0.001$), So ($F = 6.030$, $P = 0.021$), CL ($F = 16.853$, $P < 0.001$), LPI ($F = 20.337$, $P < 0.001$), LPS ($F = 14.209$, $P = 0.001$), PC ($F = 31.914$, $P < 0.001$), PE ($F = 12.559$, $P = 0.001$), PG ($F = 59.824$, $P < 0.001$), PI ($F = 23.150$, $P < 0.001$), phosphatidylserine (PS; $F = 134.871$, $P < 0.001$), LPE ($F = 15.163$, $P < 0.01$), DG ($F = 44.152$, $P < 0.001$), SQDG ($F = 10.472$, $P = 0.003$), and MGDG ($F = 12.354$, $P = 0.002$). We also found significant differences for the EA treatment factor in the concentrations of: FA ($F = 17.552$, $P < 0.001$), WE ($F = 27.347$, $P < 0.001$), Cer ($F = 4.422$, $P = 0.045$), glucosylceramides (CerG1; $F = 9.916$, $P = 0.004$), GM1 ($F = 14.835$, $P = 0.001$), phSM ($F = 27.825$, $P < 0.001$), SM ($F = 14.343$, $P = 0.001$), So ($F = 11.571$, $P = 0.002$), LPI ($F = 47.752$, $P < 0.001$), LPS ($F = 10.774$, $P = 0.003$), PA ($F = 16.878$, $P < 0.001$), PC ($F = 25.379$, $P < 0.001$), PE ($F = 17.896$, $P < 0.001$), PG ($F = 38.679$, $P < 0.001$), PS ($F = 16.869$, $P < 0.001$), monogalactosyl monoacylglycerol (MGMG; $F = 33.097$, $P < 0.001$), and MGDG ($F = 11.254$, $P = 0.002$). *Post hoc* comparisons further revealed that mice in the PTSD group exhibited significantly decreased levels of AcCa, PS, LPG, CL, LPS, PA, PG, and Co and

increased levels of LPE, LPC, and DG (PTSD *vs.* Sham, $P < 0.05$). EA treatment increased the levels of AcCa and LPG (PTSD+EA *vs.* PTSD, $P < 0.05$). Although the changes in PS, CL, LPS, PA, PG, Co, LPE, and DG induced by mSPS in the PFC were not normalized by EA treatment, other changes were, and we found no significant differences in the levels of LPE, CL, PG, and Co between PTSD+EA and Sham.

Correlation analysis showed that the time spent in the center squares in the OFT was positively correlated with levels of AcCa, FA, CL, and PS in the PFC, but negatively correlated with the level of LPE. The time spent in the open arms of the EPMT was positively correlated with levels of FA, CL, PG, phSM, and Co but negatively correlated with the level of DG. The contextual freezing time in FCR was positively correlated with the level of DG but negatively correlated with the levels of AcCa, LPG, LPS, PG, PS, Cer, phSM, and SQDG. The cued freezing time in the FCR was positively correlated with DG and LPE but negatively correlated with levels of LPG, LPS, PG, PS, and Cer (Figure 4B and Table S2). These results suggest that the levels of PS and PG in the PFC negatively correlate with the severity of PTSD, and the level of DG positively correlates with severity.

EA treatment effect: fatty acid composition

As shown in Figure 5, mSPS treatment led to a significant alteration in the fatty acyl chain profile of lipids in the hippocampus and PFC. In the hippocampus, in the PTSD group, levels of long-chain fatty acyls with 38 carbons (38C, Figure 5A) and levels of polyunsaturated fatty acyls with 4 double bonds and >6 double bonds were increased, while levels of polyunsaturated fatty acyls with 3 double bonds were decreased (Figure 5B). Notably, these changes were ameliorated by EA treatment. On the other hand, in the PFC, levels of long-chain fatty acyls with >32 carbons (38C) and levels of total unsaturated fatty acids were decreased in the PTSD group, and these changes were not attenuated after EA treatment (Figure 5C, D).

Characteristic lipid species in the brain and their correlation with PTSD-like behaviors

Lipidomic profiling at the species level revealed additional intergroup changes in the concentrations of lipids in the hippocampus and PFC. As shown in Figure 6A–C and Table S3, concentrations of 60 lipids such as PC(33:0)+H, DG(34:1e),+Na and CL(18:2/20:4/16:0/20:4)-H were decreased and those of 181 lipids such as TG(16:0/18:1/20:1)+NH4, PS(42:7p)-H, and TG(17:0/18:1/18:1)+NH4 were increased in the hippocampus in the PTSD group compared with the Sham group (Figure 6A). Concentrations of 24 lipids such as DG(34:1e)+Na, LPI(18:0)-H, and TG(18:1/18:1/18:3)+NH4 were decreased and those of 134 lipids such as PC(38:2)+H, PS(18:1/24:0)-H, and DG(18:1/22:1)+NH4 were increased in the hippocampus in the EA group compared with the Sham group (Figure 6B). EA treatment increased 47 lipids such as PC(18:0p/20:1)+HCOO, MGDG(10:4/22:6) +HCOO, and CerG1(d18:1/22:1)+H and decreased 192 lipids such as TG(18:1/18:1/18:1)+NH4, PS(42:7p)-H, and TG(17:0/18:1/18:1)+NH4 in the hippocampus in PTSD-treated mice (Figure 6C). Notably, 42 of the 241 lipid species in the hippocampus that were changed after PTSD treatment, such as Cer(d18:0+pO/24:0+O)+HCOO, PC(34:1)+H, and PS(20:4/22:6)-H, were normalized after EA treatment, suggesting that changes in these lipid species may be related to the biological effects of EA. Moreover, correlation analysis (Figure 6G) showed that the concentrations of 9

lipids such as CerG1(d18:0/24:0+O)+H, PE(18:0/18:1)+H, and PS(44:11)-H in the hippocampus were negatively correlated with the time spent in the center squares in the OFT and in the open arms of the EPMT, but positively correlated with contextual and cued freezing time in the FCR. Concentrations of 23 lipids such as CL(18:1/16:0/16:0/18:1)-H, PC(34:1)+H, and SM(d34:1)+H in the hippocampus were positively correlated with the time spent in the center squares in the OFT and in the open arms in the EPMT, but negatively correlated with contextual and cued freezing time in the FCR (see Table S4 for details), suggesting that changes in these lipid species in the hippocampus are related to the severity of PTSD.

Similar to the hippocampus, the PFC analysis revealed changes in the lipid concentrations in multiple species following mSPS and EA treatments (see Figure 6D–F and Table S5). Generally, the levels of 93 lipids such as PS(18:0/22:4)-H, PS(22:6/22:6)-H, and PS(42:7p)-H were decreased, and the levels of 37 lipids such as PC(32:0)+H, WE(21:1)+NH4, and TG(16:0/20:4/20:4)+NH4 were increased in the PFC of PTSD-treated mice compared with Sham (Figure 6D). The concentrations of 30 lipids such as PS(38:4p)-H, PS(42:7p)-H, and PC(32:0)+H were decreased and those of 201 lipids such as PE(34:2p)-H, TG(18:3/18:2/18:2)+NH4, and PC(33:0)+H were increased in EA-treated mice compared with Sham (Figure 6E). Moreover, EA treatment increased the levels of 11 lipids such as LPG(16:0)-H, DG(16:0/18:2)+NH4, and PG(40:5)+NH4, and decreased the levels of 10 lipids such as PS(18:0/22:4)-H, LPE(20:2)-H, and LPE(20:3)-H compared with the PTSD group (Figure 6F). Notably, levels of 11 of the 130 lipid molecules in the PFC changed after mSPS such as Cer(d18:0+pO/24:0+O)+HCOO, PC(34:1)+H, and PS(20:4/22:6)-H were normalized after EA treatment, suggesting that changes in these species in the PFC may be related to the biological effects of EA. Correlation analysis further showed that concentrations of LPG(16:0), DG(16:0/18:2)+NH4, PS(18:1/18:1)+H, LPG(20:4)-H, LPC(16:1p)+H, and PG(40:5)+NH4 in the PFC were negatively correlated, and concentrations of LPE(20:3)-H, LPE(20:2)-H, and DG(32:0e)+Na were positively correlated with contextual and cued freezing time in the FCR test (see Table S6 for details), suggesting that changes in these lipid species in the PFC are related to the severity of fear learning defects. Moreover, concentrations of DG(16:0/18:2)+NH4, PS(18:1/18:1)+H, and LPG(20:4)-H were positively correlated, and DG(32:0e)+Na was negatively correlated with the time spent in the open arms in the EPMT, suggesting that changes in these lipid species in the PFC are related to the severity of anxiety.

Discussion

In the present study, we performed comprehensive lipid profiling based on HPLC–MS/MS to assess the impact of EA treatment on lipidomic changes in the hippocampus and PFC of mice exposed to mSPS. We found that mSPS induced remarkable anxiety-like behaviors, fear-learning defects, and lipid changes in the brain, specifically in glycerolipids, glycerophospholipids, and sphingolipids. Moreover, the changes in lipid levels were more extensive in the hippocampus than in the PFC. Notably, early EA intervention attenuated mSPS-induced abnormal behaviors and partly normalized mSPS-induced lipid changes, notably in the hippocampus. Thus, a region-specific dysfunction of the brain lipidome may be involved in the pathogenesis of PTSD, and a region-specific regulation of the brain lipidome might partly account for the therapeutic effects of EA.

In PTSD, the hippocampus and PFC are regions of central importance due to their prominent role in both the neuroendocrine stress response and memory alterations [23, 48]. Atrophy of the hippocampus is one of the obvious pathologic changes in patients with PTSD and in animal models [49, 50]. Hippocampal dysfunction might interact with traumatic experiences to influence the etiology and maintenance of PTSD [51, 52]. Moreover, the amelioration of hippocampal structure and function was related to the alleviation of PTSD [53, 54]. On the other hand, a clinical study found that youths with PTSD had sustained decreases in gray matter volume in the right ventromedial PFC (vmPFC) and bilateral ventrolateral PFC as well as decreased ventrolateral PFC-hippocampus connectivity over time [55]. Consistent with these results, PTSD remission has been associated with expansion of frontal pole surface area and an increase in vmPFC thickness over time [25]. Moreover, a preclinical study found that the protective effects of miR-132 downregulation against behavioral impairment in rats exposed to single prolonged stress was related to a reduction in apoptosis in the PFC and an upregulation of brain-derived neurotrophic factor (BDNF) [56]. Notably, changes in the lipid composition of the hippocampus and PFC have been consistently associated with cognitive impairment and depressive-like behaviors [47, 57, 58]. Considering these results, we hypothesized that changes in the lipid composition of the hippocampus and PFC might also be associated with fear learning defects and anxiety-like behaviors in PTSD.

Glycerophospholipids and sphingolipids are important membrane lipid components that allow for the construction of a physical barrier [39, 59] and play crucial roles in regulating cell signaling [60]. Research has gradually revealed a general role for these components in neuropsychiatric diseases. Levels of PC and Cer are changed in the brain tissue of patients with bipolar disorder [61], and the relative abundances of LPC, PE, and PI in the plasma were positively correlated with the severity of depression [62, 63]. Moreover, levels of Cer are elevated in patients with major depression and bipolar disorder [64], and concentrations of SM correlate with depression and anxiety symptoms [65]. Consistent with these observations, we found that mSPS induced significant changes in glycerophospholipid and sphingolipid levels in both the hippocampus and PFC. In the PTSD group, significant increases in hippocampal levels of LPS, LPC, PI, PE, Cer, and GM1 were observed, and in PFC, significant increases in levels of LPE and LPC were observed; significant decreases were observed in hippocampal CL, LPI, and SM and in prefrontal PS, LPG, CL, LPS, PA, and PG. Notably, changes in SM, Cer, GM1, PE, CL, and LPS in the hippocampus, which were correlated with anxiety or cognitive impairment, were alleviated after EA treatment. However, in the PFC, only changes in LPG and LPC were alleviated after EA treatment. SM constitutes the vast majority of cellular sphingolipid, and can be hydrolyzed to Cer by acid sphingomyelinase (ASM). Accumulating evidences suggest that the ASM/Cer system could potentially be a novel antidepressant target, and increased activity of the ASM/Cer system might be both an effect and a cause of immune system-mediated inflammation and dysregulation of oxidative stress [66, 67]. GM1 is a key factor in maintaining the mammalian neuronal functions and avoiding neurodegeneration [68, 69] and CL plays a role in the regulation of cellular energy metabolism and mitochondrial function [70]. LPC homeostasis is an endogenous mediator of myelin injury [71]; LPC levels are elevated in the cerebrospinal fluid of patients with multiple sclerosis [72] and in the brain following ischemia [73]. LPS and LPG represent an immunomodulatory effect [74, 75]. PE is involved in the modulation of membrane fluidity

and properties that protect the cell under conditions of oxidative stress [76]. Thus, mSPS-induced anxiety-like behaviors and fear learning defects are related to an imbalance in levels of specific glycerophospholipids and sphingolipids in both the hippocampus and PFC. Lipidomic analyses revealed that 42 species, mainly belonging to the CL, PC, CerG1, PS, and SM series, were changed after mSPS in the hippocampus, while 11 species, mainly belong to the LPG and LPE series, were changed in the PFC, all of which were alleviated after EA. These results further indicate that the modulation of glycerophospholipid and sphingolipid levels after EA was brain area-specific, and that the lipid regulatory effect of EA was greater in the hippocampus than in the PFC.

In addition to the disturbance in levels of membrane lipid classes observed in the pathophysiology of PTSD, a growing body of evidence indicates that abnormalities in glycerolipid and fatty acyl compositions may also be involved [40, 77]. The present study showed that mSPS increased the levels of glycerolipids in both the hippocampus (DG and TG) and the PFC (DG). However, only levels of TG, which were positively correlated with anxiety-like behaviors, were normalized after EA treatment, suggesting that the modulation of TG in the hippocampus is at least partially responsible for the protective effects of EA against PTSD.

In the brain, AcCa compounds functionally alter and stabilize membranes, thereby improving mitochondrial function and enhancing antioxidant activity [78, 79]. Recent studies have demonstrated that AcCa levels in the plasma of depressed subjects are lower than those of healthy controls and that these alterations correlate with the severity of the patients' depressive symptoms [80]. Our data show that mSPS induced a significant decrease in AcCa levels in both the hippocampus and PFC, which was negatively correlated with contextual freezing time and was normalized after EA treatment. Thus, a reduction in brain AcCa is involved in the pathophysiology of stress-induced fear learning defects and recovery of AcCa levels is associated with the protective effects of EA observed in this study.

The biophysical properties of lipids are influenced by the chain length (number of carbons) and the degree of saturation of the constituent fatty acyls [81]. Modulation of the composition of the fatty acyl chains and degree of saturation of membrane lipids can potentially affect neuronal functioning, at least in part through altered function of membrane-bound proteins [58, 82]. The present study found that changes in the composition of fatty acyls in the hippocampus attributable to mSPS were ameliorated after EA treatment. However, the more extensive changes observed in the PFC were not normalized after EA treatment, suggesting that fatty acyl composition in the PFC is more vulnerable to mSPS and does not recover as quickly as that in the hippocampus. Notably, we also found that after mSPS, the concentration of Co(Q9) was lowered in both the hippocampus and PFC and was effectively normalized after EA treatment. CoQ is a redox-active molecule that plays a fundamental role in mitochondrial energy generation and functions as a potent endogenous antioxidant [83]. Our results indicate that a disturbance of the brain antioxidant system is involved in the pathogenesis of PTSD and that Co(Q9)+NH4 is potentially a molecular target for the treatment of PTSD.

Recent work has previously reported an influence of acupuncture treatment on both depressive-like behaviors and the lipidomics of the liver [84]. However, the neuroprotective effects of EA are closely related to its action parameters and action time, and precedents exist for suspecting that early intervention using EA might be an effective strategy for preventing the development of PTSD symptoms [54, 85]. Previous studies have found that EA stimulation (2/15 Hz) applied at the GV20 (“Bai hui”) point enhanced motor performance recovery and BDNF expression in rats with cerebral infarction [86]. EA treatment has also been shown to reduce glutamate toxicity and exert antiapoptotic effects in a rat stroke model [87, 88]. Importantly, our previous work found that early EA intervention applied at the GV20 point with the same frequencies (2/15 Hz) prevents PTSD-like behaviors in a rat model of PTSD [28]. Consistent with these results, the present study indicates that early intervention using EA administered for seven continuous days exerts protective effects on experimental PTSD in mice and mobilizes changes in brain lipid compositions, mainly in the hippocampus. Thus, we speculate that different frequencies and current intensity of EA might induce different neuromodulation effects by influence the composition of lipids in the brain. Neither did we investigate the effects of EA at different time points, nor the relationships between different parameters of EA and the composition of lipids in the brain, which should be the goals of future research.

Taken together, our results indicate that lipidomic changes in the PFC and hippocampus may be a key aspect of the pathogenesis of PTSD. Importantly, the functions of these lipids are mainly focused on anti-inflammation, energy metabolism, oxidative stress, and neuroprotection, which may partly explain the neuroprotective effect of EA. The observation of a correlation between levels of oxidative stress, inflammation, or levels of neurotrophic factors in the hippocampus or PFC might provide further insight into this hypothesis. The present study found that levels of AcCa, CL, and Co were decreased after EA while that of LPC was increased in both hippocampus and PFC. However, changes in LPS and DG showed an opposite trend in the hippocampus and PFC, which might relate to the divergent structures and functions of these brain regions. The precise mechanisms of brain area-specific modulation after EA and the effects of specific lipid species on PTSD-like behaviors require further investigation. Furthermore, although all mice in this study were exposed to isoflurane inhalation anesthesia, we cannot rule out an anesthetic effect on our results due to the regulatory effect of isoflurane on brain lipidomics and its potential influence on brain oxidative stress, inflammation, and levels of neurotrophic factors [89–91].

In summary, our study revealed distinct changes in lipid composition within the hippocampus and PFC in mice after exposure to mSPS. We further confirmed that the hippocampus is more sensitive to mSPS-induced lipid modulation than is the PFC and showed that EA is effective in normalizing the changes in lipid composition in the hippocampus, notably changes in sphingolipids and glycerophospholipids. Based on these observations, we conclude that the distribution of lipids across the hippocampus and PFC may dictate regional susceptibility to stress and explain the neuromodulatory effects of EA. However, the influence of behavioral testing and different EA parameters on brain lipids, as well as the precise mechanism through which EA regulates lipid composition, require further investigation.

Abbreviations

PTSD: posttraumatic stress disorder; EA: electroacupuncture; PFC: prefrontal cortex; mSPS: modified single prolonged stress; HPLC–MS/MS: high performance liquid chromatography–tandem mass spectrometry; OFT: open field test; EPMT: elevated-plus maze test; FCR: fear conditioning response test; MTBE: methyl tert-butyl ether; SD: standard deviation; ANOVA: one-way analysis of variance; DG: diglyceride; TG: triglyceride; SM: sphingomyelin; Cer: ceramides; GM1: gangliosides; CL: cardiolipin; LPG: lysophosphatidylglycerol; LPI: lysophosphatidylinositol; LPS: lysophosphatidylserine; PA: phosphatidic acid; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; AcCa: acylcarnitine; SQDG: sulfoquinovosyldiacylglycerol; MGDG: monogalactosyldiacylglycerol; Co: coenzyme; So: sphingosine; FA: fatty acid; DGDG: digalactosyldiacylglycerol; LPC: lysophosphatidylcholine; PC: phosphatidylcholine; WE: wax esters; CerG2GNAc1: simple Glc series; phSM: sphingomyelin; PS: phosphatidylserine; CerG1: glucosylceramides; ASM: acid sphingomyelinase; NSM: neutral sphingomyelinase

Declarations

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Code Availability

Not applicable

Authors contributions

CZ, FX, QS, SX, TZ, XM, LY and CL were responsible for collecting the data and behavioral evaluation. ZP and HW financed and designed the study and supervised the data collection and analysis, CZ analyzed the data and wrote the first draft with ZP and FX. All other authors provided data, reviewed the results, and contributed to the final draft of the report.

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Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Ethics Approval

The experimental procedures of this study were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Use and Protection Committee of the Fourth Military Medical University (KY20193003).

Consent to Participate

Not applicable

Consent of Publication

All authors read and approved the final manuscript.

Conflict of Interest

The authors have declared that no competing interest exists

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Figures

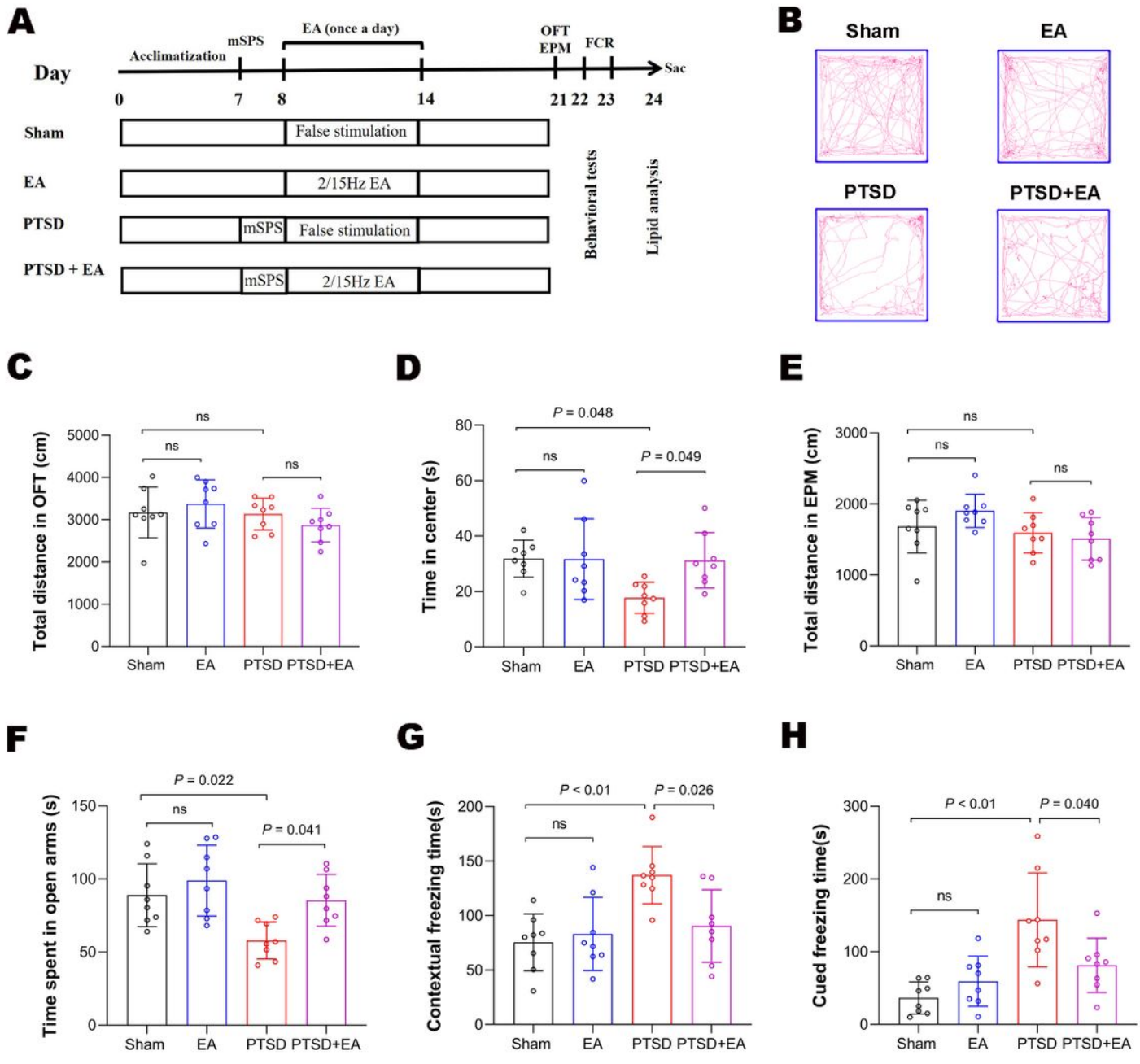

Figure 1

Early intervention using electroacupuncture (EA) ameliorates posttraumatic stress disorder (PTSD)-like behaviors in model mice treated with a modified single prolonged stress (mSPS) procedure. (A) The experimental design. After one week of adaptation, animals were administered mSPS or a sham treatment, then EA (2/15 Hz, 1.0 mA) or false stimulation was administered once a day for 30 min per day from days 8 to 14. Behavioral alterations were assessed from days 21 to 23, then mice were sacrificed (sac) for tissue collection. (B) Representative real-time movement traces in the open field test (OFT) for each group. (C) Quantification of the total distance traveled during the OFT. (D) Time spent in the center squares in the OFT. (E) Quantification of the total distance traveled in the elevated-plus maze test

(EPMT). (F) Time spent in the open arms in the EPMT. (G) Freezing time measured in the contextual fear response test. (H) Freezing time measured in the cued fear response test.

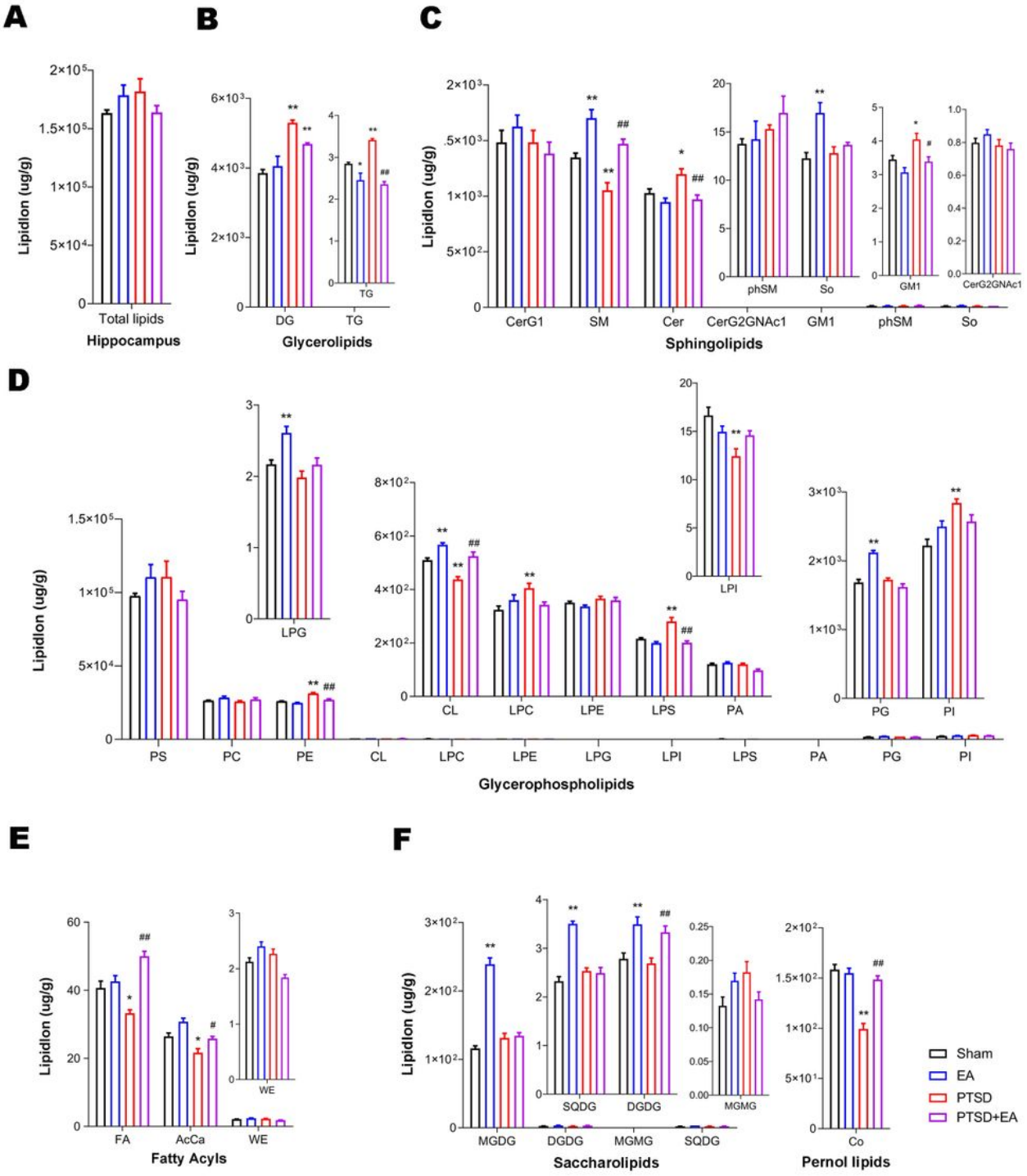

Figure 2

Groupwise alterations in the lipidomic profiles in the hippocampus. (A) Total lipid concentration, (B) glycerolipids, (C) sphingolipids, (D) glycerophospholipids, (E) fatty acyls, (F) saccharolipids and prenol lipids. DG, diglyceride; TG, triglyceride; CerG, glucosylceramides; SM, sphingomyelin; Cer, ceramides; GM1,

gangliosides; phSM, phytosphingosine sphingomyelin; So, sphingosine; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; FA, fatty acid; AcCa, acylcarnitine; WE, wax esters; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; MGMG, monogalactosylmonoacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; Co, coenzyme; PTSD, posttraumatic stress disorder; EA, electroacupuncture; *, $P < 0.05$ vs. Sham; **, $P < 0.01$ vs. Sham; #, $P < 0.05$ vs. PTSD; ##, $P < 0.01$ vs. PTSD.

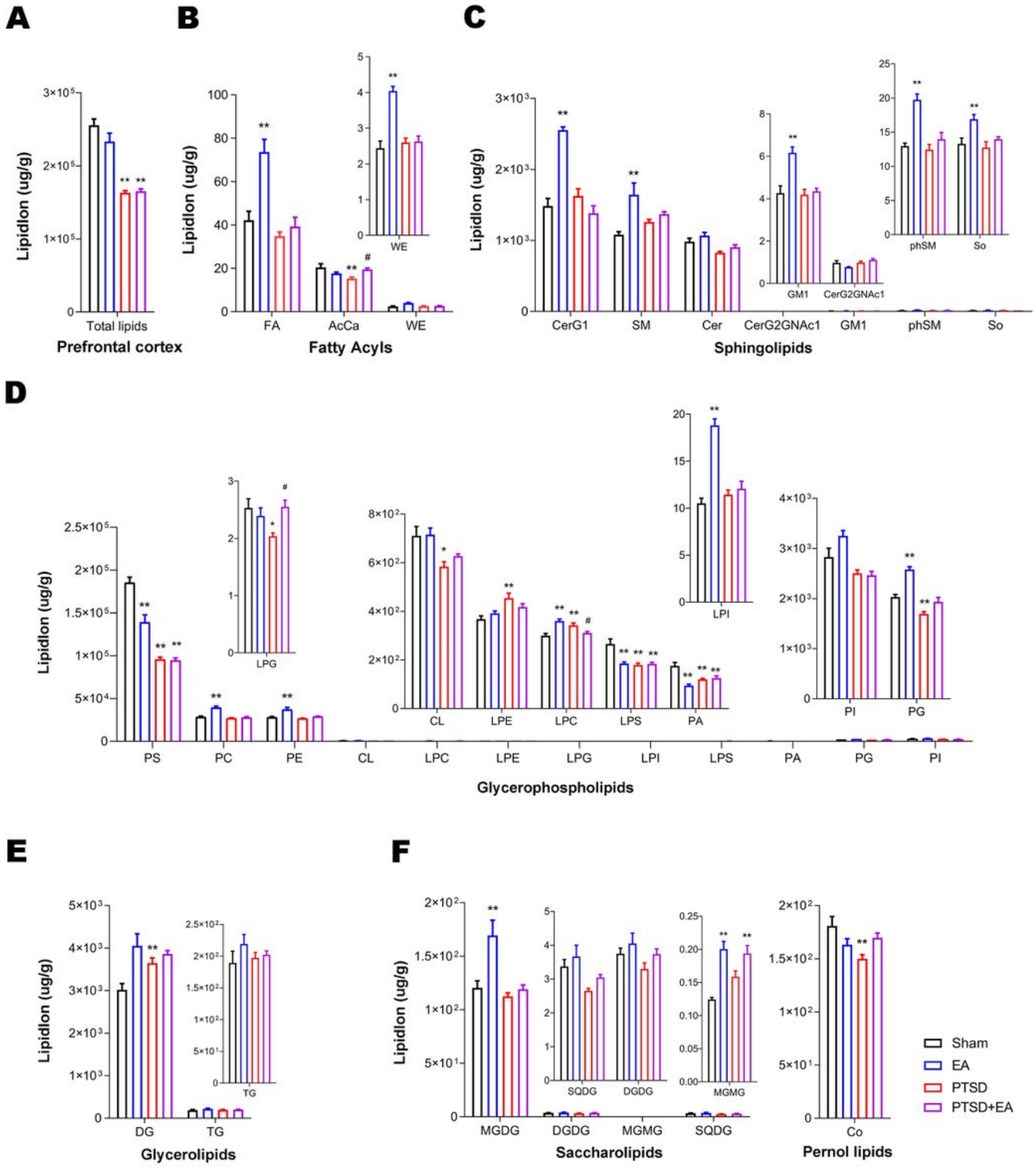

Figure 3

Groupwise alterations in the lipidomic profiles in the prefrontal cortex (PFC). (A) Total lipid concentration, (B) fatty acyls, (C) sphingolipids, (D) glycerophospholipids, (E) glycerolipids, (F) saccharolipids and prenol lipids. FA, fatty acid; AcCa, acylcarnitine; WE, wax esters; CerG, glucosylceramides; SM, sphingomyelin; Cer, ceramides; GM1, gangliosides; phSM, phytosphingosine sphingomyelin; So, sphingosine; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL,

cardiolipin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; DG, diglyceride; TG, triglyceride; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; MGMG, monogalactosylmonoacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; Co, coenzyme; PTSD, posttraumatic stress disorder; EA, electroacupuncture; *, P < 0.05 vs. Sham; **, P < 0.01 vs. Sham; #, P < 0.05 vs. PTSD.

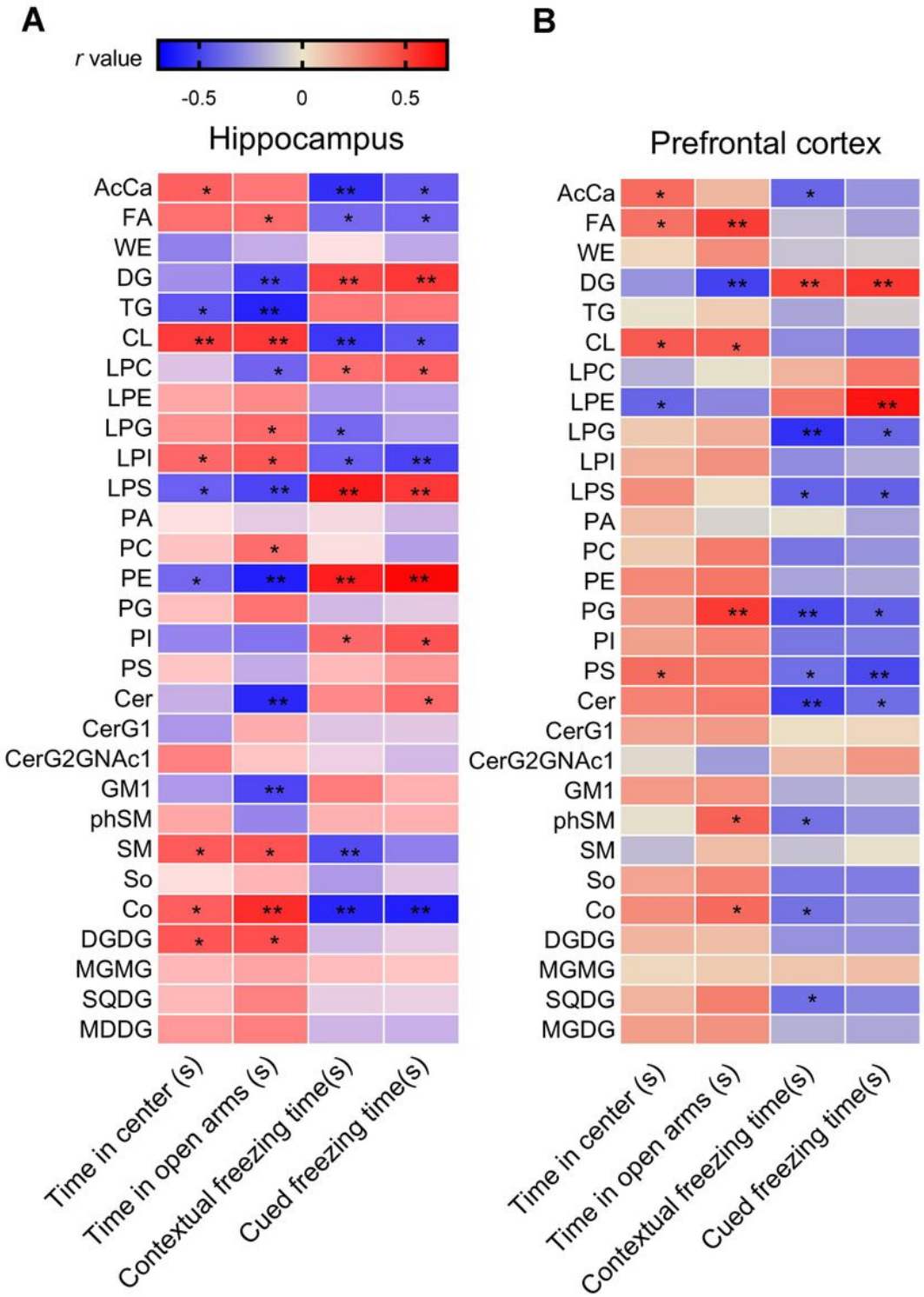

Figure 4

Analysis of correlations between PTSD-like behaviors and lipid levels in (A) the hippocampus and (B) the PFC, using Pearson's correlation coefficient. *, P < 0.05; **, P < 0.01.

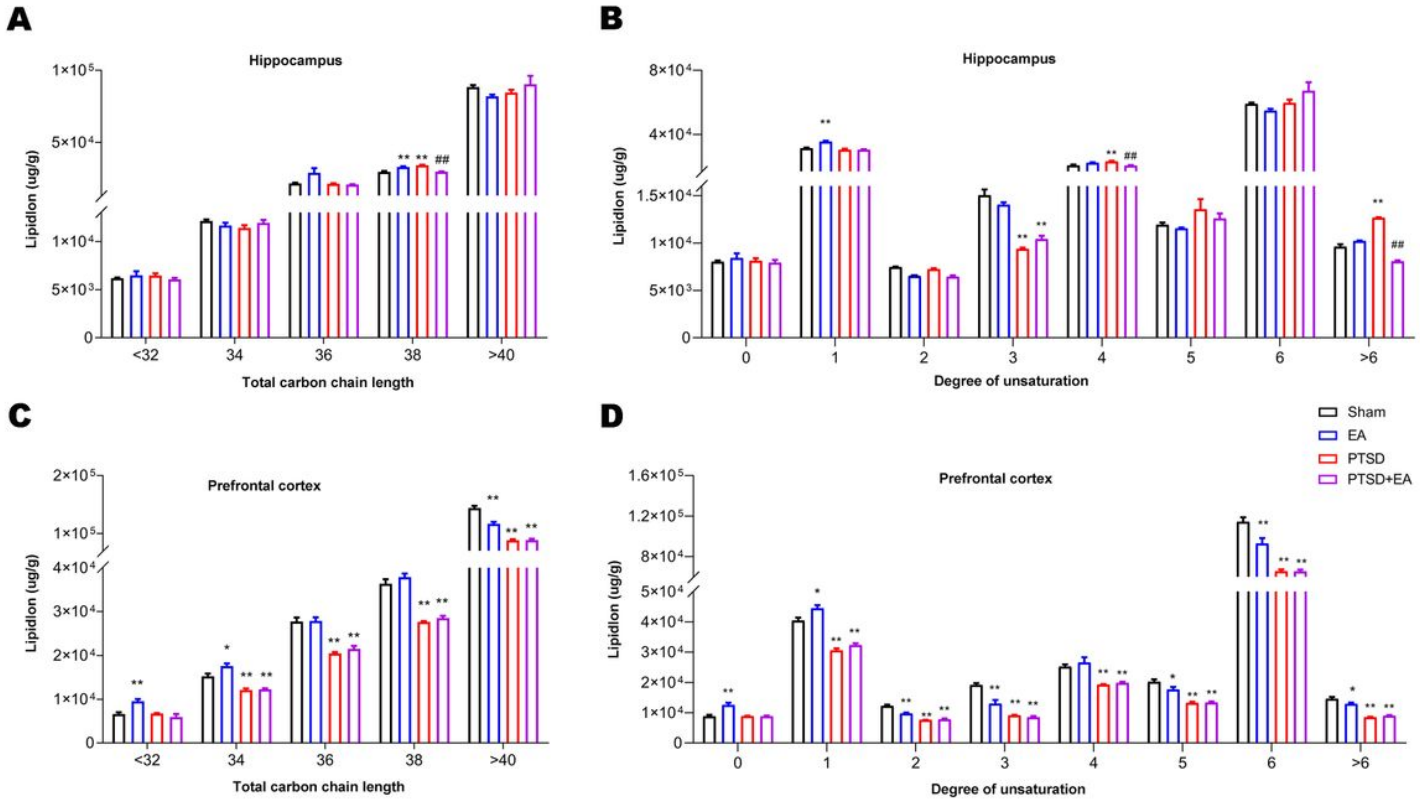

Figure 5

Groupwise alterations in fatty acid composition in the hippocampus and PFC. Results of analysis of fatty acyl composition by (A, C) chain length (number of carbons) totals and (B, D) degree of unsaturation totals. PTSD, posttraumatic stress disorder; EA, electroacupuncture; *, P < 0.05 vs. Sham; **, P < 0.01 vs. Sham; #, P < 0.05 vs. PTSD; ##, P < 0.01 vs. PTSD.

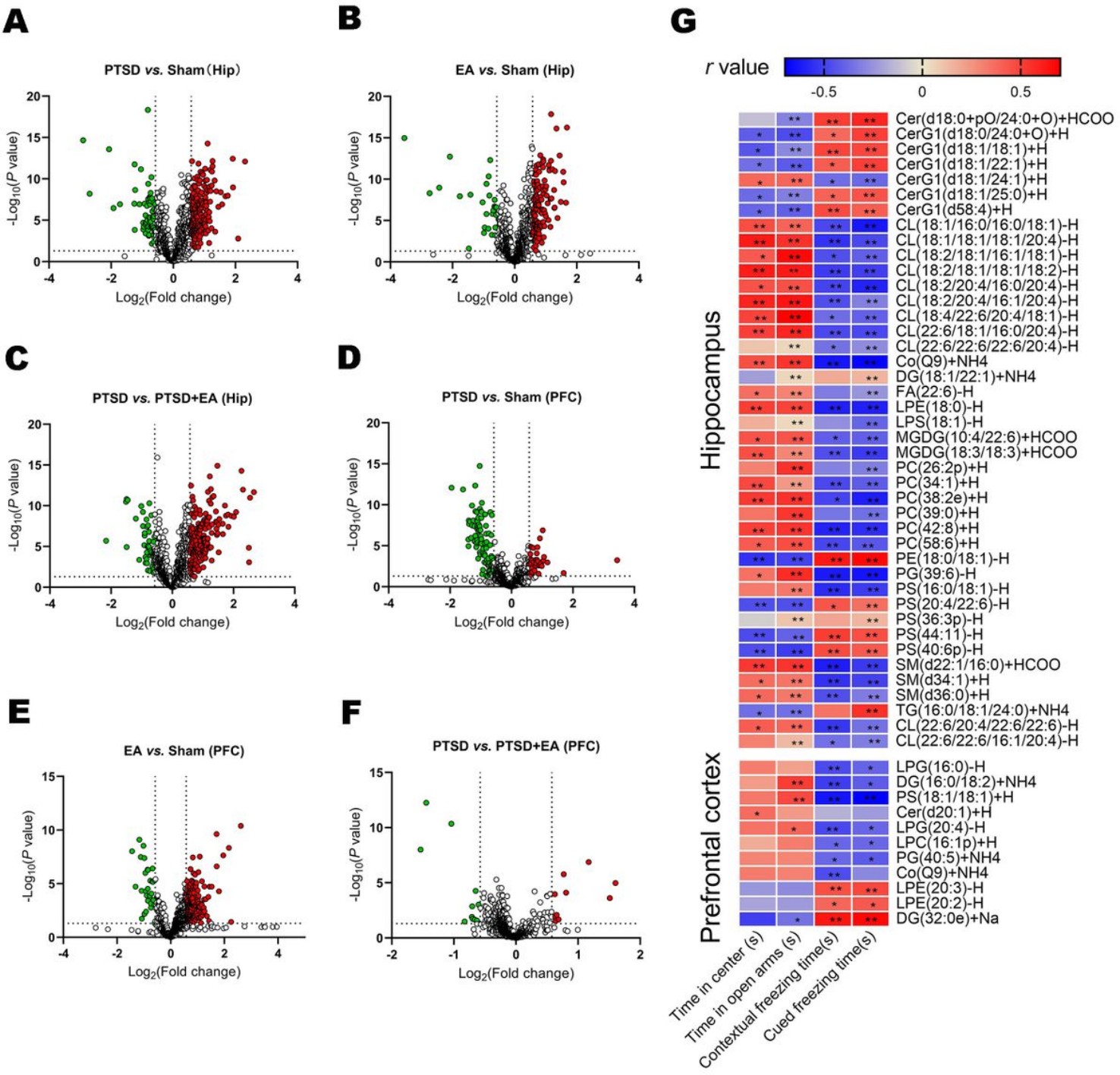

Figure 6

Groupwise characterization of lipid species and their correlations with PTSD-like behaviors. (A–C) Volcano map showing decreased (green dots) and increased (red dots) lipid species comparing the PTSD group and Sham, the EA group and Sham, and the PTSD group and the PTSD+EA group in the hippocampus. (D–F) Same comparisons for the PFC. (G) Correlation between PTSD-like behaviors and levels of lipid species in the hippocampus and PFC. The behavioral correlations were analyzed using Pearson's correlation coefficient. Hip, hippocampus; PFC, prefrontal cortex; *, $P < 0.05$; **, $P < 0.01$.

Supplementary Files

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