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Protection of p-Coumaric acid against chronic stress-induced neurobehavioral deficits in mice via activating the PKA-CREB-BDNF pathway

Yanqun Cao [a], Hao Chen [a], Yinna Tan [b], Xu-Dong Yu [a], Chuli Xiao [a], Yin Li [a], James Reilly [c], Zhiming He [a,*], Xinhua Shu [a,c,d,*]

[a] The Brain Cognition and Brain Disease Branch, Pu Ai Medical School, Shaoyang University, Shaoyang 422000, China
[b] Anesthesiology department, The Affiliated Nanhua Hospital, Hengyang Medical School, University of South China, Hengyang 421000, China
[c] Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Glasgow G4 0BA, UK
[d] Department of Vision Science, Glasgow Caledonian University, Glasgow G4 0BA, UK

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ABSTRACT

There is a body of evidence to suggest that chronic stress modulates neurochemical homeostasis, alters neuronal structure, inhibits neurogenesis and contributes to development of mental disorders. Chronic stress-associated mental disorders present common symptoms of cognitive impairment and depression with complex disease mechanisms. P-coumaric acid (p-CA), a natural phenolic compound, is widely distributed in vegetables, cereals and fruits. p-CA exhibits a wide range of health-related effects, including anti-oxidative-stress, anti-mutagenesis, anti-inflammation and anti-cancer activities. The current study aims to evaluate the therapeutic potential of p-CA against stress-associated mental disorders. We assessed the effect of p-CA on cognitive deficits and depression-like behavior in mice exposed to chronic restraint stress (CRS); we used network pharmacology, biochemical and molecular biological approaches to elucidate the underlying molecular mechanisms. CRS exposure caused memory impairments and depression-like behavior in mice; p-CA administration attenuated these CRS-induced memory deficits and depression-like behavior. Network pharmacology analysis demonstrated that p-CA was possibly involved in multiple targets and a variety of signaling pathways. Among them, the protein kinase A (PKA) - cAMP-response element binding protein (CREB) - brain derived neurotrophic factor (BDNF) signaling pathway was predominant and further characterized. The levels of PKA, phosphorylated CREB (pCREB) and BDNF were significantly lowered in the hippocampus of CRS mice, suggesting disruption of the PKA-CREB-BDNF signaling pathway; p-CA treatment restored the signaling pathway. Furthermore, CRS upregulated expression of proinflammatory cytokines in hippocampus, while p-CA reversed the CRS-induced effects. Our findings suggest that p-CA will offer therapeutic benefit to patients with stress-associated mental disorders.

1. Introduction

It is well established that stress is a major environmental factor that influences the physical and psychological health of individuals and leads to various types of disease, particularly stress-associated mental disorders [1], common symptoms of which include mood dysfunction and memory impairment. These disorders are complex, serious and not uncommon, with their global occurrence on the rise in recent years. Given the limited therapeutic options, there is an urgent need to screen active compounds that have the potential to treat, with fewer side effects, the mood disorders and memory impairment associated with stress-related mental disorders [1,2].

Stress can modulate neuronal structure and function, resulting in abnormal emotion, behavior and cognition [1]. It can induce dysfunction of the hypothalamic-pituitary-adrenal axis, trigger oxidative damage and cause inflammation and neurotransmission abnormalities, all of which may facilitate progression of mental disorders [1,3]. The hippocampus is a brain region of central importance in this context as it not only participates in stress responses but is also involved in the regulation of memory and mood state [4]. Chronic stress inhibits neurogenesis in

* Corresponding authors.
E-mail addresses: 40003@hnsyu.edu.cn (Z. He), xinhua.Shu@gcu.ac.uk (X. Shu).

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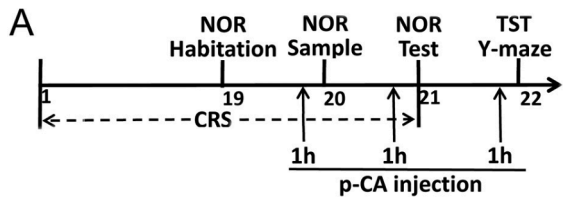

Fig. 1. Schematic of the experimental design showing the timeline of drug injection and behavior tasks. CRS, chronic restraint stress; NOR, novel object recognition; TST, tail suspension test; p-CA, p-coumaric acid.

the hippocampus and reduces its volume. Stressed rodents demonstrated microglial activation in the CA1 and CA3 hippocampal regions and showed increased production of proinflammatory cytokines, which lead to impaired neurogenesis [3,4]. Increased oxidative stress and impaired antioxidative capacity have been reported in the hippocampus of chronically stressed rodents [5]. Chronic stress has been widely reported to cause dysregulation of neurotransmitters in rodent hippocampus [4]. Decreased expression, release and transmission of serotonin have been demonstrated in the hippocampus of rats under chronic stress [6,7]; other studies have shown that, in rat hippocampus, chronic stress promotes release of glutamate and expression of glutamate receptors while decreasing the clearance and metabolism of glutamate [8,9]. These pathological effects result in impaired memory.

P-Coumaric acid (p-CA), a natural phenolic compound, is widely present in vegetables, fruits and cereals that have been reported to exert beneficial effects, such as anti-oxidative, anti-inflammatory and anti-cancer properties. Additionally, p-CA has been shown to play powerful neuroprotective roles. For example, p-CA treatment enhances hippocampal neurogenesis, reduces hippocampal neuronal death, activates brain-derived neurotrophic factor signaling and improves long-term potentiation (LTP) in hippocampal slice [10–12]. Moreover, p-CA treatment also alleviated 5-S-cysteinyl-dopamine, mutant copper-zinc superoxide dismutase 1 (SOD1) or amyloid beta (Aβ) induced neurotoxicity in models of neurodegenerative disease, e.g., Alzheimer's disease [13], Parkinson's disease [14] and amyotrophic lateral sclerosis [15]. Importantly, p-CA also alleviates $AlCl_3$-, D-galactose-, scopolamine-, lipopolysaccharide (LPS)- and corticosterone-induced memory impairment, as well as LPS- or corticosterone-induced depression-like behaviors [12,16–20]. However, the effects of p-CA on stress-related mental disorders, such as stress-induced memory impairment and depression-like behavior, have not been investigated.

In the present study, we used chronic restraint stress (CRS) rodent model, a classic model for stress-related mental disorder, to investigate the effects of p-CA on memory deficits and depression-like behavior. Additionally, network pharmacology and biochemical analyses were performed to predict and verify the underlying mechanisms in the hippocampus.

2. Material and methods

2.1. Animals

Seven-week old male Institute of Cancer Research (ICR) mice (30±2 g) were obtained from the Hunan SJA Laboratory Animal Co., Ltd., Hunan, China. These mice were paired, housed with food and water and maintained on a 12-h light–dark cycle (lights on at 7:00 AM) at a constant temperature of 25±2 °C and humidity of 55±10 %. Prior to the treatment, the mice were handled 5 min per day by the experimenter for 5 consecutive days. All animal experiments followed the Guidance for the Care and Use of Laboratory Animals, University of South China (Project license number SYXK2020–0002).

2.2. Animal treatment

Animals were randomized into three groups (10/group): control; chronic restraint stress (CRS); and CRS+*p*-CA groups. Animals in both CRS and CRS+*p*-CA groups were exposed to 21 consecutive days of restraint stress according to the previous description [21], while animals in the control group were kept unrestrained in the cage. The CRS+*p*-CA group received intraperitoneal injections of p-CA, dissolved in 0.9 % saline containing 10% Tween 80 (vehicle) at a dosage of 75 mg/kg body weight for 3-consecutive days (days 19–21). The dose of 75 mg/kg body weight was chosen based on our previous rodent studies and those of others [17–20]. Both the control and CRS groups received intraperitoneal injections of vehicle for 3-consecutive days (Fig. 1).

2.3. Behavior tests

2.3.1. Novel object recognition (NOR) task

The NOR task consisted of three phases: habituation, sampling and test phase, conducted on, respectively, days 19, 20 and 21; details of the procedure and apparatus are described in a previous study [22]. On day 19, habituation phase commenced 1 h after the p-CA treatment by introducing each mouse to an empty Plexiglas chamber (30 cm × 30 cm × 65 cm) and allowing free exploration for 10 min. On day 20, the sampling phase commenced 1 h after the p-CA treatment when the mice were introduced to the training chamber in which were two identical objects and allowed to freely explore it for 10 min. On day 21, 24 h after the sampling phase, the test phase commenced 1 h after the p-CA treatment. Mice were reintroduced to the training chamber in which one of the two identical objects had been removed and replaced by a novel object and allowed to freely explore for 5 min,. The total distance traveled and the time spent exploring both objects was recorded by, respectively, behavior-tracking software (Anymaze 6.16) or a trained observer in a double-blind manner. Memory performance was expressed as the discrimination index (DI), which refers to the difference between time spent in exploring a new object and a familiar object divided by the total time exploring both objects.

2.3.2. Y-maze test

The Y-maze task was conducted on day 22, 1 h after p-CA treatment; procedure and apparatus is described in a previous study [22]. Briefly, each mouse was placed into the Y-maze and allowed to move freely for a 5-min period. The behavior of the mice was recorded by digital camera, with the total number of entries into the arms and the alteration ratio then analyzed and calculated by a trained observer in a double-blind manner.

2.3.3. Tail suspension test (TST)

The TST was conducted on day 22, 2 h after the Y-maze task; details of the procedure are described in a previous study [21]. Briefly, each mouse was suspended about 58 cm above the ground for a 6 min test period by adhesive tape placed approximately 1 cm from the tail tip. During the 6 min testing, the immobility time was scored for the last 4 min by an experienced observer in a double-blind manner.

2.4. Bioinformatic analysis

2.4.1. Screening the target genes of p-CA and stress-related mental disorders

The structures of canonical SMILES strings of p-CA were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/, accessed on 12 January 2023), the obtained strings were imported into three public databases including Swiss Target Prediction Platform (http://www.swisstargetprediction.ch/, accessed on 12 January 2023, probability>0), similarity ensemble approach (SEA, https://sea.bkslab.org/, accessed on 12 January 2023)(select all targets) and Super-PRED (https://prediction.charite.de/, accessed on 12 January 2023) to

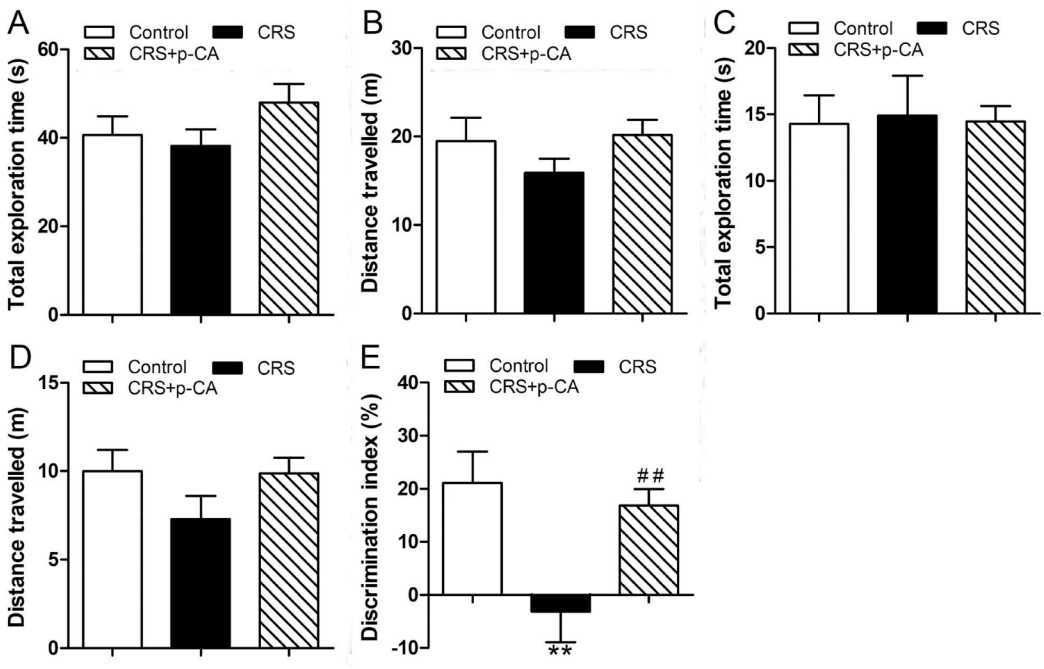

Fig. 2. Effect of p-CA administration on memory impairments induced by CRS in the NOR task. During the sampling phase, the total exploration time (A) and the total distance traveled (B) for three groups. During the test phase, the total exploration time (C), the total travelling distance (D), and the discrimination index (E) for the three groups. $^{**}p$ <0.01, CRS group VS Control group; $^{\#\#}p$ <0.01, CRS+*p*-CA group VS CRS group (n = 10). CRS, chronic restraint stress; NOR, novel object recognition; p-CA, p-coumaric acid.

collect potential targets. Disease-related targets were collected from GeneCards databases (https://www.genecards.org/, accessed on 15 January 2023) using "Stress-related mental disorders" as the keyword.

2.4.2. Construction of venn diagram and protein-protein interaction (PPI) network

Target genes of both p-CA and stress-related mental disorders were imported into the Venn diagram (https://bioinfogp.cnb.csic.es/tools/venny/, accessed on 15 January 2023), where the overlapping targets were obtained and visualized. To further identify the core potential targets, overlapping targets were imported into the STRING database (https://cn.string-db.org/, accessed on 17 January 2023). The screening condition was set up as "Homo sapiens", the medium confidence was > 0.4, and unconnected target protein nodes were hidden. The network graphics in tab separated values (TSV) format were imported into the Cytoscape (https://cytoscape.org/, accessed on 17 January 2023) for construction of a PPI network.

2.4.3. Enrichment analysis

We used the "clusterProfiler" package (https://bioconductor.org/packages/ release/bioc/html/clusterProfiler.html, accessed on 20 January 2023) in the R platform (https://www.rstudio.com/, accessed on 20 January 2023) to analyze gene ontology (GO) enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment based on the potential target genes. The top ten GO terms and top twenty KEGG pathways were visualized.

2.4.4. Molecular docking

The 3D structure of p-CA was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/.accessed on 26 January 2023), imported into the OpenBabel3.1.1 software (https://openbabel.org/wiki/Main_Page) and converted to the mol2 format. AutoDock4.2.6 software (https://autodock.scripps.edu/) was utilized for hydrogen addition, deletion of water, designation as ligand and retaining the torsional bonds. The 3D structure of the core targets was retrieved from the Protein Data Bank (PDB) database (https://www.pdbus.org/accessed on 26 January 2023). The AutoDock4.2.6 tool was used to add hydrogen atoms, remove the water, and set it as a receptor. The docking results were analyzed and visualized via the Pymol and Discovery Studio.

2.5. Western blotting

To asess and avoid the effect of behavioral testing on target protein expression in this study, another cohort of mice received the same treatment as described in Section 2.2, without performing the behavioral tasks. These mice were sacrificed 1 h after the final p-CA intraperitoneal injection on day 22, then the whole hippocampus tissues were dissected and immediately frozen at -80 °C. The hippocampus tissues were homogenized and lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Shanghai, China) containing protease inhibitor cocktail (Thermo Fisher Scientific, Shanghai, China) for 30 min, then centrifuged (12,000 g for 15 min at 4 °C). The supernatants were collected, and sample protein concentration was measured. 50 μg protein samples were separated in 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5 % skimmed milk powder then incubated with primary antibodies including PKA catalytic subunit (Cat no: 27,398–1-AP, Proteintech, Rosemont, IL 60,018, USA, 1:5000), CREB (Cat no: 4820S, Cell Signaling Technology, Danvers, Massachusetts, USA, 1:1000), p-CREB (Cat no: 28,792–1-AP, Proteintech, Rosemont, IL 60,018, USA, 1:1000) or BDNF (Cat no: ab226843, Abcam, Cambridge, UK, 1:1000), β-actin (Cat no: 81,115–1-RR, Proteintech, Rosemont, IL 60,018, USA, 1:5000), then incubated with the secondary antibody (Cat no: SA00001–2, Proteintech, Rosemont, IL 60,018, USA, 1:5000). Signals of targeted proteins were visualized with the ChemiDoc XRSImaging system.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the hippocampus using TRIzol reagent. cDNA was synthesized using the PrimeScript RT reagent kit

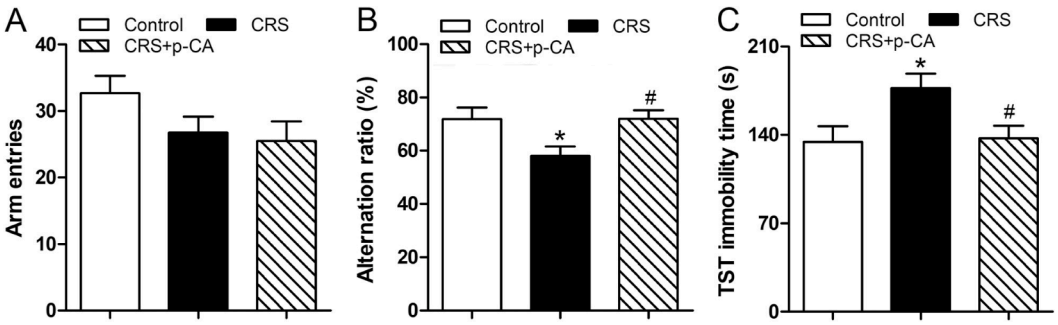

Fig. 3. Effect of p-CA administration on CRS-induced memory impairments and depression-like behavior. In the Y-maze task, the number of arm entries (A) and alternation ratio (B) for the three groups are shown. (C) The immobility time for the three groups in the TST is shown. Date are expressed as mean ±SEM ($n = 10$ mice/group). *p <0.05, CRS group VS Control group; #p <0.05, cRS+p-CA group VS CRS group. CRS, corticosterone; p-CA, p-coumaric acid.

following the manufacturer's instruction. QRT-PCR assay was completed using the StepOnePlus RT–PCR System based on the manufacturer's protocol. The mRNA level of target genes was calculated using the $2^{-\Delta\Delta Ct}$ formula and normalized to *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) gene. Sequences of primers for qRT-PCR are listed in Table S1.

2.7. Data analysis

All data were analyzed using the Sigma Plot 12.5 software and presented as mean ± standard error of the mean (SEM). The significance of the difference between groups was assessed using One-way analysis of variance (ANOVA). Fisher LSD method was used as a post-hoc test to check for difference in variance. P-value < 0.05 was defined as a significant difference.

3. Results

3.1. p-CA attenuated memory impairment of stress-related mental disorders in CRS mice

To evaluate the functional role of p-CA in stress-related mental disorders, we investigated whether p-CA administration reversed CRS-induced memory impairment using NOR and Y-maze tasks. In the NOR task, during sample phases, all groups showed no significant difference in total exploration time (Fig. 2A, $F(2,25) = 1.6$, $p > 0.05$) and travelling distance (Fig. 2B, $F(2,25) = 1.257$, $p > 0.05$). During the test phase, all groups also showed no significant difference in total exploration time (Fig. 2C, $F(2,25) = 0.0205$, $p > 0.05$); Although the travelling distance of the CRS mice was shorter than that of the control and CRS+p-CA mice, there were no significant differences between each group (Fig. 2D, $F(2,25)=1.767$, $p > 0.05$). However, the CRS mice showed a significantly decreased discrimination index when compared to that of the control group, while p-CA administration counteracted this change (Fig. 2E, $F(2,25) = 6.579$, $p < 0.01$).

In the Y-maze task, all groups showed no significant difference in the number of arm entries (Fig. 3A, $F(2,24) = 2.016$, $p > 0.05$). However, compared with the control mice, CRS exposure significantly decreased the alteration ratio, while co-treatment with p-CA significantly increased the alteration ratio, compared with mice exposed to CRS alone (Fig. 3B, $F(2,24) = 4.352$, $p < 0.05$).

3.2. p-CA administration reversed depression-like behavior in CRS mice

We also investigated the effect of p-CA CRS-induced despair behavior in the TST. CRS exposure significantly increased the immobility time compared to that of control mice, while co-treatment with p-CA significantly counteracted the CRS-induced effect (Fig. 3C, $F(2,25) = 4.549$, $p < 0.05$).

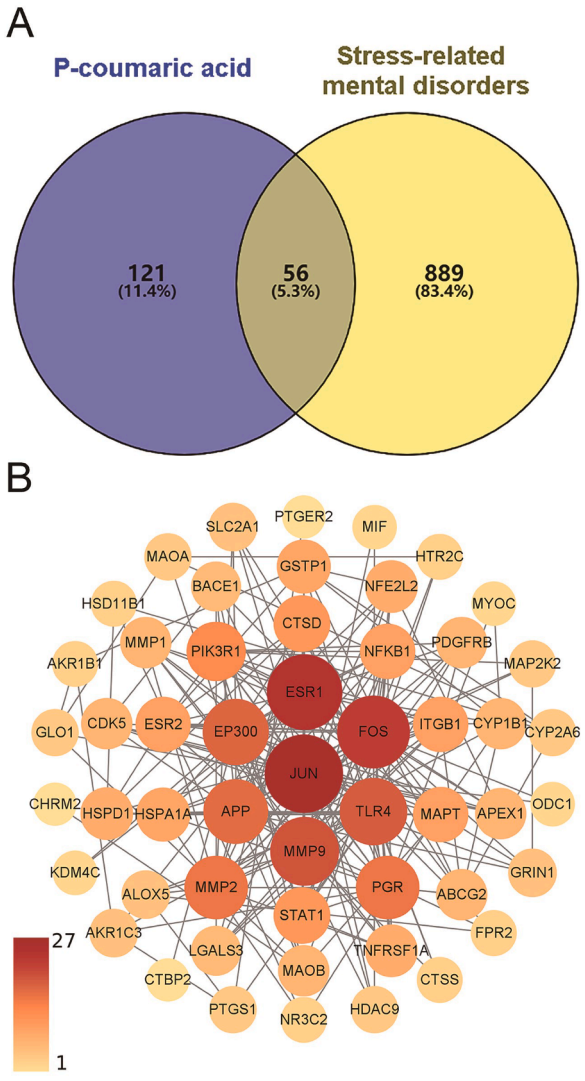

Fig. 4. (A) Shared target genes of p-coumaric acid, associated with stress-related mental disorders. (B) Protein-protein interaction (PPI) network of these targeted genes. The sizes and colors of the nodes are representative of the degree values; the nodes of large and darker color of red signifies a higher degree value.

3.3. Overlapping targets of p-CA and stress-related mental disorders

A total of 83 promising targets were predicted and identified from the SEA database. Moreover, 88 and 39 targets were retrieved from,

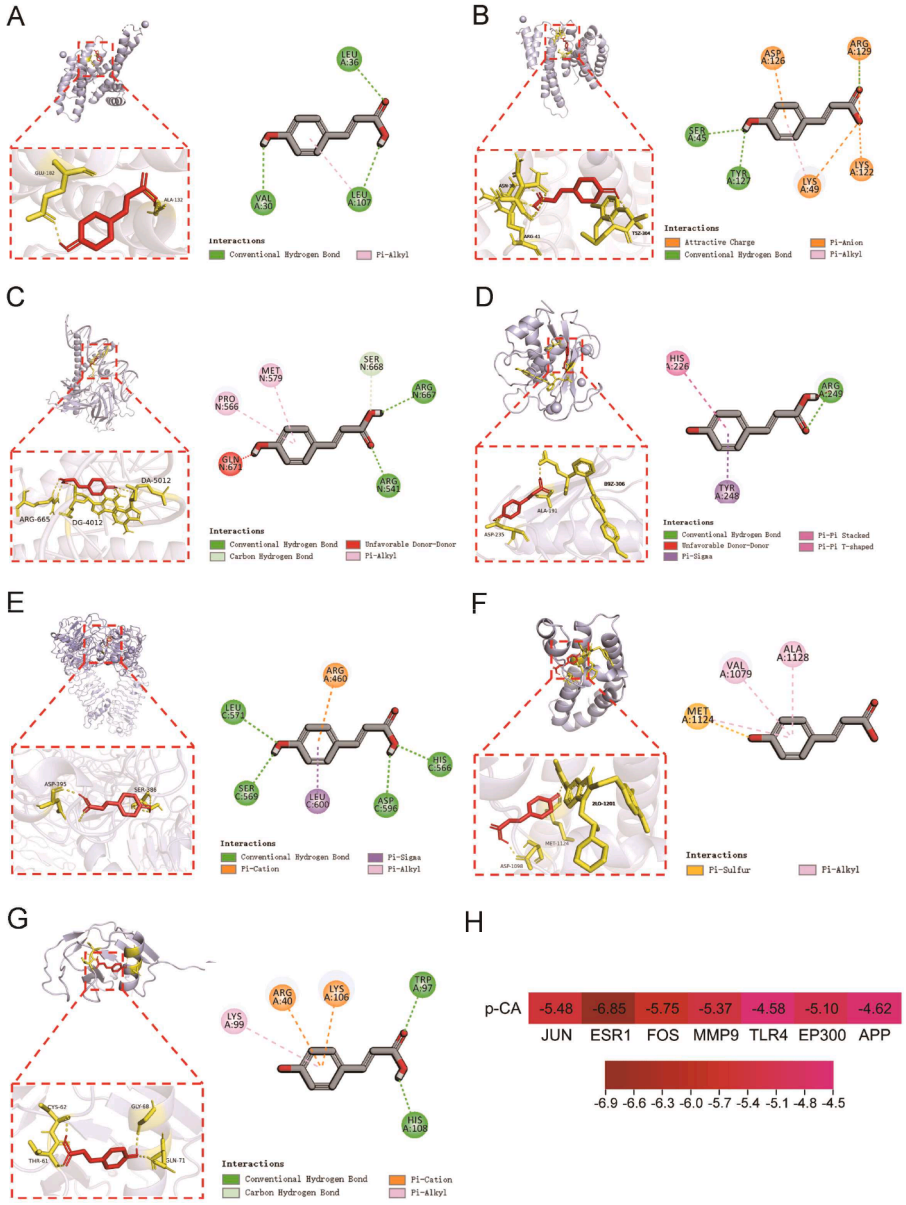

Fig. 5. Molecular docking pattern of p-CA and its targets. (A) p-CA-JUN. (B) p-CA-ESR1. (C) p-CA-FOS. (D) p-CA-MMP9. (E) p-CA-TLR4. (F) p-CA-EP300. (G) p-CA-APP. (H) the heat map of the molecular docking scores of p-CA and its targets. p-CA, p-coumaric acid.

respectively, the SuperPred and Swiss Target Prediction databases. After deleting the duplicated targets, 177 p-CA targets were identified (Table S2). We also collected 947 targets for stress-related mental disorders from the GeneCards database (Table S3). In order to explore the relationship between p-CA and stress-related mental disorders, the 177 p-CA targets and 945 disease targets were uploaded to the Venn program; 56 overlapping targets were obtained (Table S4).

We imported the above 56 targets into the STRING database to construct a PPI network containing 53 nodes and 216 edges (Fig. 4A), except for vasopressin V1b receptor (AVPR1B), transient receptor potential cation channel subfamily M member 2 (TRPM2) and fat mass and obesity-associated protein (FTO), which were not linked to this network (Fig. 4B). In the network, the hub node was regarded as a potential core target and node size correlated positively with the degrees. Based on the topological properties, we use "degree value" as a screening parameter to measure potential core targets in the entire network. The results suggested that JUN, estrogen receptor 1 (ESR1), FOS, matrix metallopeptidase (MMP9), toll-like receptor 4 (TLR4), E1A-associated protein p300 (EP300) and amyloid precursor protein (APP) were ranked higher (degree >16) (Table S4), denoting them as potential core targets for the treatment of stress-related mental disorders with p-CA.

To investigate the underlying mechanism of p-CA's protective role against chronic stress-induced mental disorders, we performed molecular docking using p-CA as a ligand and the top 7 core targets (JUN, ESR1, FOS, MMP9, TLR4, EP300 and APP) as receptors. The results revealed that p-CA interacted with JUN at Val-30, Leu-36 and Leu-107 residues, with ESR1 at Ser45, Lys49, Lys122, Asp126, Tyr127 and Arg129 residues, with FOS at Arg541, Pro566, Met579, Gln617, Arg667 and Ser668 residues, with MMP9 at His226, Tyr248 and Arg249 residues, with TLR4 at Arg460, His566, Ser569, Leu571, Asp596 and Leu600 residues, with EP300 at Val1079, Met1124 and Ala1128 residues, and with APP at Arg40, Trp97, Lys99, Lys106 and His-108 residues (Fig. 5).

3.4. P-CA target-associated function and signaling pathways

To further reveal the functions and mechanisms of p-CA targets in the treatment of stress-related mental disorders, we performed GO and

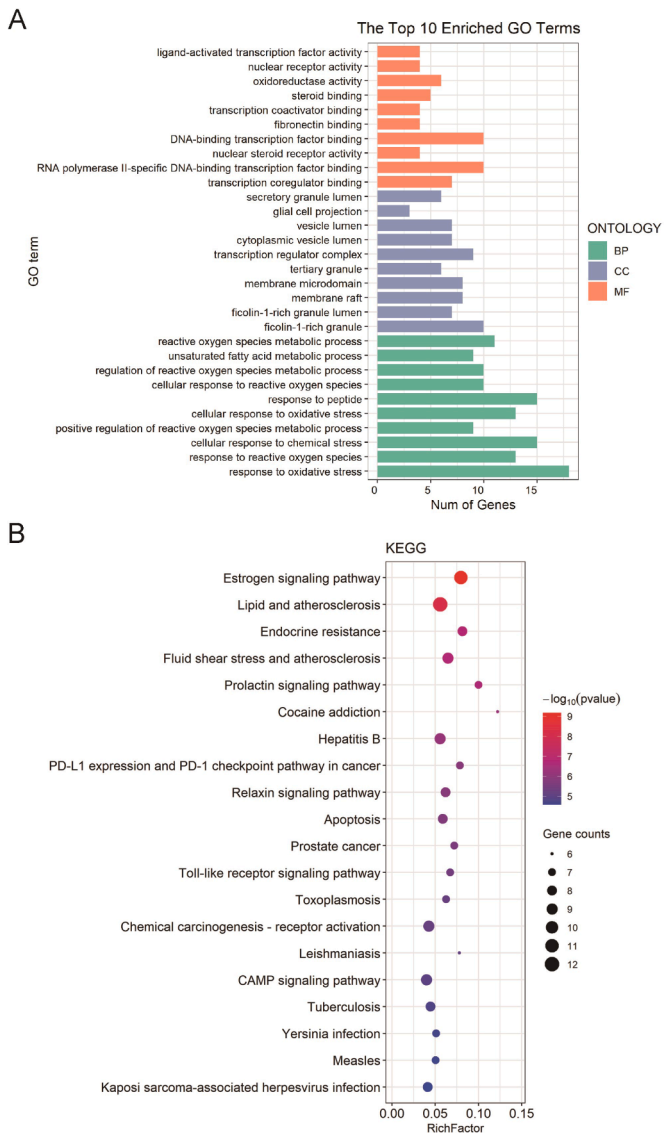

Fig. 6. (A) Top 10 gene ontology (GO) terms of potential core targets. The vertical axis indicates the number of enriched genes, and the horizontal axis indicates three categories. From top to bottom are molecular function (MF) terms, cell component (CC) terms and biological process (BP) terms. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of potential core targets, showing the top 20 signaling pathways.

KEGG enrichment analyses of core targets using the R Studio software. A total of 870 GO terms were identified, including 751 terms in biological processes (BP), 36 terms in cell components (CC), and 83 terms in molecular functions (MF). The top 10 terms of BP, CC and MF are shown in Fig. 6A. The core targets mainly functioned in steroid binding and nuclear (steroid) receptors that are associated with stress-related mental disorders [23]. Meanwhile, the top targets predominantly mediated transcription factor/coactivator/coregulator binding, which may regulate expression of genes associated with development of chronic stress-induced mental disorders [24,25]. These core targets participated primarily in the vesicle lumen, membrane raft, cytoplasmic vesicle lumen, and secretory granule lumen, where receptor-mediated signal transductions are involved in the development of chronic stress induced mental disorders [26]. In terms of MF, the core targets were mainly involved in the responses to oxidative stress, which is strongly associated with chronic stress-induced mental disorders [27]. We further explored p-CA-mediated mechanisms using the KEGG pathway analysis and found that some of the top 10 pathways including the estrogen signaling pathway, the Prolactin signaling pathway, the Toll-like receptor signaling pathway and the cAMP signaling pathway, are strongly associated with the occurrence and development of chronic stress induced mental disorders [28–31].

3.5. P-CA treatment upregulated the PKA-CREB-BDNF pathway in the hippocampus of CRS mice

The protein kinase A (PKA) - cAMP response-element binding protein (CREB) - brain derived neurotrophic factor (BDNF) pathway plays a critical role in the pathogenesis of stress-associated mental disorders, such as anxiety, depression and dementia [31]. Network pharmacology analysis also demonstrated p-CA targeting of the PKA pathway (Fig. 4 and 6B). We examined the expression of PKA, CREB and BDNF in the hippocampus of control, CRS and CRS+ p-CA mice. Compared to those of control mice, the levels of PKA catalytic subunit ($F(2,12)= 34.803$), phosphorylated CREB (p-CREB) ($F(2,12)= 51.422$) and BDNF ($F(2,12)= 69.619$) were significantly decreased in the CRS mice ($p < 0.05$, Fig.7 and S1), while co-treatment with p-CA significantly increased the levels of these proteins compared to those of CRS mice ($p < 0.05$). However, there was no difference in total CREB protein between individual mouse groups (Fig. 7A and D, $F(2,12) = 0.0400$, $p > 0.05$).

3.6. P-CA reversed CRS-induced inflammation in the hippocampus

Chronic restraint stress has been shown to cause hippocampal inflammation, including microglial activation and generation of proinflammatory cytokines, in rodents (3). Expression of proinflammatory cytokine genes in the hippocampus of the mouse groups was detected by qRT-PCR. The results demonstrated that the mRNA levels of hippocampal IL-1β ($F(2,12)= 84.509$), IL-6 ($F(2,12) = 22.452$) and TNFα ($F(2,12) = 60.538$) were markedly increased in CRS mice, compared to that of control mice (Fig. 8, $p < 0.001$); co-treatment with p-CA significantly decreased the levels of the three genes compared to that of CRS mice (Fig. 8, $p < 0.001$). These observations suggested that p-CA attenuated CRS-induced hyperactivation of hippocampal inflammation in mice.

4. Discussion

In the current study, we aimed to evaluate the protective effect of p-CA against CRS-induced mental disorders in mice. We observed that CRS induced memory impairment in the NOR and Y-maze tasks. Moreover, CRS inactivated the PKA-CREB-BDNF signaling pathway and upregulated expression of proinflammatory cytokines. P-CA counteracted CRS-induced effects, possibly via multiple targets and associated signaling pathways, including the PKA-CREB-BDNF signaling pathway.

Stress is one of the crucial risk factors for mental disorders such as anxiety and depression (1). CRS is a classic stress procedure to induce mental disorders in rodents [32]. Previous studies have demonstrated that CRS induces anxiety, cognitive impairment and depressive behaviors in rodents [33,34]. Consistently, our present study found that CRS led to a decreased discrimination index in the NOR task and decreased alteration ratio in the Y-maze task, suggesting cognitive deficit. It was important to exclude the influence of nonspecific responses (locomotor activity and exploration level) on the memory performance. Therefore, the distance traveled, total exploration time and arm entries were examined in, respectively, the NOR and Y-maze tasks. Our results showed that CRS did not induce any significant difference in locomotor activity and exploration level between individual mouse groups, indicating that CRS-induced memory deficit was not influenced by the nonspecific responses. Furthermore, CRS also caused significantly increased immobility time, which has implications for depression.

Previous studies have demonstrated that p-CA alleviates scopolamine- (an antagonist of cholinergic muscarinic receptors) induced memory impairment in rats [12], ameliorates cognitive deficits and

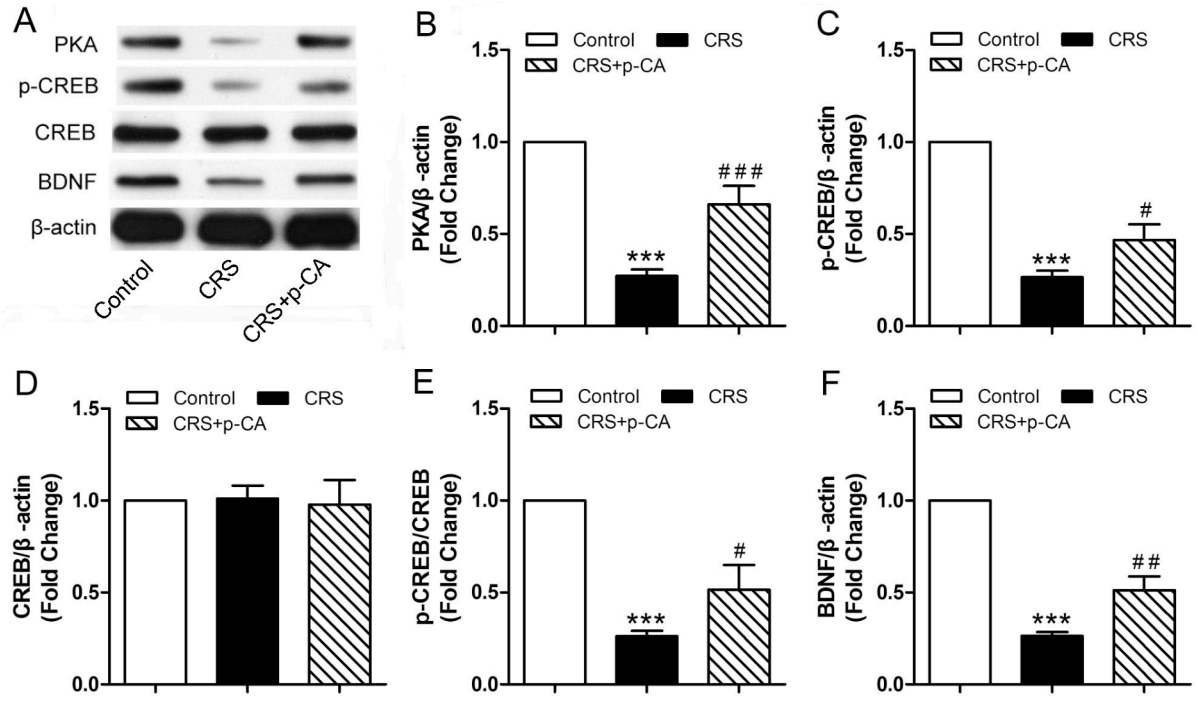

Fig. 7. Effect of p-CA treatments on CRS-induced downregulation of the PKA-CREB-BDNF signaling pathway in mouse hippocampus. (A) Representative immunoblots of hippocampal PKA catalytic subunit, p-CREB, CREB, BDNF. (B-F) Quantification for immunoblots of PKA (B), p-CREB (C), CREB (D) p-CREB/CREB, BDNF (F) normalized to β-actin, and p-CREB normalized to CREB (E). Data were expressed as mean ±SEM ($n = 5$ mice/group). $^{***}p < 0.001$, CRS group VS control group; $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$, CRS+*p*-CA VS CRS group. CRS, chronic restraint stress; p-CA, p-coumaric acid.

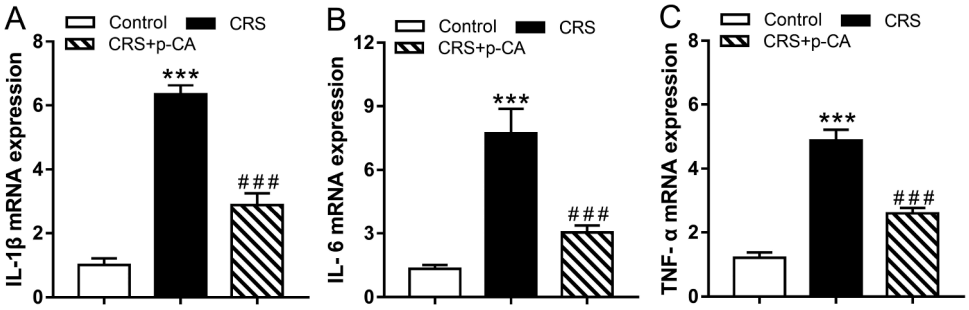

Fig. 8. Effect of p-CA treatment on CRS-induced upregulation of proinflammatory cytokines in mouse hippocampus. The mRNA levels of IL-1β (A), IL-6 (B) and TNF-α (C) detected by qRT-PCR. Data are expressed as mean ±SEM ($n = 5$ mice/group) $^{***}p < 0.001$, CRS group VS control group; $^{###}p < 0.001$, CRS+*p*-CA group VS CRS group. CRS, chronic restraint stress; p-CA, p-coumaric acid.

mitigates depression-like behaviors in AlCl3-exposed rats (16) and in corticosterone-administrated mice [19]. Additionally, p-CA has been shown to alleviate learning and memory deficits in lipopolysaccharide (LPS)- or D-galactose-treated mice [17,18], and in rats with transient middle cerebral artery occlusion [10]; in an onset LPS-injection-induced acute depression model, p-CA reversed depression-like behaviors in a forced swim test, TST and sucrose splash test [20]. In the current study we also found that memory impairments induced by CRS were attenuated by p-CA treatment in the NOR and Y-maze tasks without influencing the locomotor activity and exploration level, further supporting the memory-improving effect of p-CA; furthermore, p-CA also mitigated CRS-induced depression-like behavior.

Network pharmacology has been widely used to predict drug targets and the underlying functional mechanisms [35]. Network pharmacology analysis showed that p-CA had 56 targets associated with stress-mediated mental disorders. The seven core targets – JUN, ESR1, FOS, MMP9, TLR4, ESP300 and APP – are well-documented to be involved in the development of stress-related mental disorders [28,30,36–40]. Molecular docking also predicted that p-CA physically interacts with these core targets, possibly directly mediating their functions. Biological Gene ontology function analysis showed that these core targets play a critical role in response to oxidative stress. P-CA has been shown to have the capacity of counteracting oxidative damage in a variety of disease models [16,18,41]. Early studies reported that CRS-induced cognitive deficits are associated with oxidative stress [42]. Therefore, it is worth examining the role of p-CA against oxidative stress in alleviating cognitive impairment in the CRS mice. KEGG analysis predicted the core targets are involved in multiple functional signaling pathways, of which the estrogen signaling pathway, toll-like receptor signaling and cAMP signaling pathway have been shown to play critical roles in chronic stress associated mental disorders [28,30,31].

In the physiological condition, cAMP as the second messenger activates PKA, subsequently phosphorylates CREB and upregulates expression of multiple downstream targeting genes, including BDNF [31]. Stress has been shown to play a critical role in modulating the PKA level in rodent models and in the postmortem brain of suicide individuals: the PKA/CREB/BDNF signaling pathway is disrupted in the prefrontal cortex and hippocampus of suicidal subjects [43]. Chronic social defeat

stress (CSDS) can induce downregulation of CREB and BDNF in mouse raphé nuclei of the midbrain [44] and overexpression of BDNF in the serotonergic neurons protected mice against CSDS, possibly via enhancing both the production of neuroprotective hormones and the promotion of neurogenesis in the hippocampus [45]. Recent studies also show that CRS significantly lowers the levels of phosphorylated CREB (pCREB) and BDNF in mouse hippocampus [46,47]. We also confirmed that the levels of PKA, pCREB and BDNF were markedly decreased, while p-CA treatment counteracted CRS-induced effects. 5-HT1A receptor (5-HT1AR) is suggested to play an important role in stress-associated mental disorders [48]. 5-HT1AR is believed to couple with an inhibitory G-protein, resulting in inhibition of adenylyl cyclase and decreased cAMP generation, which can downregulate expression of PKA [48,49]. Previous studies have reported that CRS causes a decrease in 5-HT1AR expression and cAMP level in mouse hippocampus [50,51]. Therefore, it would be worth further examination of hippocampal 5-HT1AR expression and cAMP level in our CRS mice and the effect of p-CA treatment on 5-HT1AR expression and cAMP level.

Chronic stress plays an important role in the development and progression of mental disorders [3]. Previous studies reported that CRS induced expression of proinflammatory cytokines (e.g. IL-1β, IL-6 and TNFα) and microglial activation, possibly via activation of TLR 4-mediated signaling pathways in rodent hippocampus [52–54]. Here we also confirmed upregulation of hippocampal proinflammatory cytokines in CRS mice and showed that p-CA treatment markedly downregulated expression of proinflammatory cytokines. Recent studies have demonstrated that protection of p-CA against diabetic nephropathy or hepatic fibrosis is mediated by inhibition of TLR4 signaling pathway [55,56]. KEGG analysis also showed that p-CA-associated core targets were possibly involved in TLR signaling pathway. Therefore, it is reasonable that p-CA inhibits the TLR signaling pathway, although this requires further confirmation. Previous studies have shown that inflammation can significantly downregulate expression of BDNF, which contributes to cognitive deficit and mental disorders [57,58]. It is possible that p-CA suppressed CRS-induced inflammation, upregulated BDNF expression and reversed CRS-caused neurobehavioral deficits.

5. Conclusion

This study indicates that p-CA ameliorates CRS-induced memory impairment and depression-like behavior in mice. The protection of p-CA against stress-associated mental disorders is possibly mediated by multiple targets and signaling pathways, of which the PKA/CREB/BDNF pathway and the TLR signaling pathway are predominant. The results suggest that p-CA has therapeutic potential for stress-associated mental disorders. This proof-of-concept study will benefit a clinical trial of p-CA treatment in patients with these disorders.

Author contributions

Y.C., H.C., Y.T., X.D.Y., C.X., Y.L. performed the experiments; Z.H. and X.S. supervised the project; X.D.Y and X.S. analyzed the data. X.D.Y., J.R., X.S. drafted the manuscript.

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Supplementary materials

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