Journal of Ginseng Research 47 (2023) 662–671

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: https://www.sciencedirect.com/journal/journal-of-ginseng-research

Research Article

Discovery and validation of PURA as a transcription target of 20(S)-protopanaxadiol: Implications for the treatment of cognitive dysfunction

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ARTICLE INFO

Article history:
Received 9 January 2023
Received in revised form
23 March 2023
Accepted 24 April 2023
Available online 29 April 2023

Keywords:
Drug affinity responsive target stability
Transcription factors
PURA
20(S)-protopanaxadiol
Cognitive dysfunction

ABSTRACT

Background: 20(S)-protopanaxadiol (PPD), a ginsenoside metabolite, has prominent benefits for the central nervous system, especially in improving learning and memory. However, its transcriptional targets in brain tissue remain unknown.
Methods: In this study, we first used mass spectrometry-based drug affinity responsive target stability (DARTS) to identify the potential proteins of ginsenosides and intersected them with the transcription factor library. Second, the transcription factor PURA was confirmed as a target of PPD by biolayer interferometry (BLI) and molecular docking. Next, the effect of PPD on the transcriptional levels of target genes of PURA in brain tissues was determined by qRT-PCR. Finally, bioinformatics analysis was used to analyze the potential biological features of these target proteins.
Results: The results showed three overlapping transcription factors between the proteomics of DARTS and transcription factor library. BLI analysis further showed that PPD had a higher direct interaction with PURA than parent ginsenosides. Subsequently, BLI kinetic analysis, molecular docking, and mutations in key amino acids of PURA indicated that PPD specifically bound to PURA. The results of qRT-PCR showed that PPD could increase the transcription levels of PURA target genes in brain. Finally, bioinformatics analysis showed that these target proteins were involved in learning and memory function.
Conclusion: The above-mentioned findings indicate that PURA is a transcription target of PPD in brain, and PPD upregulate the transcription levels of target genes related to cognitive dysfunction by binding PURA, which could provide a chemical and biological basis for the study of treating cognitive impairment by targeting PURA.

1. Introduction

Transcription factors (TFs) are key cellular components that guide gene expression, recognizing specific DNA sequences to control chromatin and transcription [1]. There is increasing evidence that dysregulation of transcription factor function is one of the causes of human neurodegenerative diseases [2]. For instance, alterations in transcription factor function are key contributors to Alzheimer's Disease (AD) progression and pathogenesis, thus making transcription factors potential therapeutic targets for AD [3]. In addition, transcription factors targeting adult neurogenesis in the hippocampus have been investigated to reduce the memory impairment associated with AD [4]. Previous studies have highlighted the specific role of Nuclear receptor subfamily 4, group A, member 2 (Nr4a2) in hippocampal synaptic plasticity and memory formation, and discussed whether the dysregulation of this transcription factor can lead to hippocampal synaptic

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https://doi.org/10.1016/j.jgr.2023.04.007

dysfunction, suggesting that Nr4a2 may be a new synaptic therapeutic target related to cognitive dysfunction [5]. Therefore, transcription factors play a critical role in cognitive dysfunction and are expected to be effective therapeutic targets.

Ginseng is the dried root and rhizome of *Panax ginseng Meyer*, a perennial herb of the family *Araliaceae*, has a long history of application as a traditional Chinese medicine to improve learning and memory in eastern Asian [6]. Ginsenosides, the main active components of ginseng, are mainly composed of protopanaxadiol-type and protopanaxatriol-type saponins [7]. There is increasing evidence that they have a variety of neuroprotective effects, such as improving cognitive dysfunction and enhancing learning and memory [8]. For example, the active components of ginsenoside, Rg1 and Rb1, have been demonstrated to promote cholinergic neurotransmission in the cholinergic nervous system and improve learning and memory function [9]. Yang et al. showed that cognitive impairment caused by insulin resistance could be improved by ginsenoside Rb1 via Cdk5/p35-NMDAR-IDE pathway [10].

Previous studies have demonstrated that gut bacteria and liver can metabolize prototype ginsenosides into rare saponins or aglycogens *in vivo*, and the biological activity of rare saponins or aglycones is usually higher than that of prototype ginsenosides [11,12]. As an aglycone of ginsenosides, 20(S)-protopanaxadiol (PPD) has been proved to penetrate the blood brain barrier (BBB) in several studies [13,14] and play a pharmacological role in the central nervous system (CNS). For example, Lu et al. found that PPD attenuated memory impairment induced by scopolamine in mice by modulating cholinergic and antioxidant systems, suggesting that it may be a candidate compound to prevent memory loss in some neurodegenerative diseases, such as AD [15]. Moreover, it has been proven that PPD can protect mice from memory impairment caused by chronic sleep deprivation (CSD) in mice [16]. Numerous studies have explained the possible mechanism of PPD in neuroprotection, but there are few studies on their direct targets in the brain, especially the transcription targets. Whereas the identification of small molecular compound targets is of great significance for understanding the mechanism of potential therapeutic drugs.

Recently, many new target discovery techniques have been discovered with the development and application of bioinformatics and proteomics. Drug affinity responsive target stability (DARTS) is a new method for target discovery, which is especially good at screening the targets of small molecular compounds without any structural modifications [17]. DARTS is a reliable method for the detection of natural product targets that is selected by exploiting the phenomenon of protease protection. When a small molecule drug combines with the target protein to stabilize the structure of the target protein, it can effectively prevent the target protein from being degraded by protease [18]. After screening by DARTS method, the targets are further validated by proteomics, such as liquid chromatography-mass spectrometry (LC-MS) [19]. The DARTS method has been widely used to reveal drug-macromolecular interactions due to its simplicity and high efficiency.

Using DARTS based on LC-MS, we first screened three transcription factors as potential target proteins of ginsenosides in brain tissues, including Pur-alpha (PURA), Khsrp (KSRP), and Pur-beta (PURB). Considering that the interaction between PURA and PURB was reported in the literature [20], we then verified the relationship between them through STRING online database (https://string-db.org/) and biolayer interferometry (BLI) technology. PURA was subsequently selected for further study. Then, we used BLI to detect the direct interaction between PURA and ginsenosides. The BLI results indicated that PPD, a metabolite of ginsenosides, had a strong direct interaction with PURA. The affinity of PPD and PURA was further verified by BLI kinetic analysis, molecular docking and amino acid site-directed mutagenesis. The effect of PPD on the transcriptional levels of PURA target genes was determined by qRT-PCR *in vivo*. Finally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed by DAVID service to verify the potential biological features of PURA, PURB, five target genes of PURA, and proteins directly interacted with them analyzed by STRING database.

2. Methods

2.1. Extraction of high-purity ginseng total saponins (GTS)

High-purity GTS were extracted as previously described [21]. Rb1,Rg1, F1,Re, Rh2, Rh1, Rd, compound K (CK), PPD, 25-OH PPD, 20(S)-Protopanaxatriol (PPT) standards (purity≥98%) and PPD (purity ≥95%) used *in vivo* experiments were purchased from Shanghai Yuanye Biotechnology Co., Ltd.

2.2. Experimental animals

Male adult Sprague-Dawley rats, weighing 180-220 g, were from the Laboratory Animal Center of Nanjing University of Chinese Medicine. Rats were maintained at 22 ± 2 °C on a 12 h/12 h light/dark cycle with free access to water and food. Tissues were collected from mice deeply anesthetized with sodium pentobarbital. All animal manipulations were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine and were performed in accordance with the Guidelines for animal rearing and care established by the Chinese Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. We used the minimum number of animals needed to obtain consistent data. And the study protocol was approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine (Permit Number: 202012A042).

2.3. Drug affinity responsive target stability (DARTS)

The protocol for the implementation of DARTS was modified from Pai et al. [22]. The brain tissues of rats were taken under pentobarbital sodium anesthesia and homogenized in RIPA lysis buffer (pH = 7.4). And detailed procedures are described in Supplementary Methods. Finally, the samples were analyzed by label-free LC-MS.

2.4. Label-free LC-MS and data analysis

Label-free LC-MS was used to carry out quantitative proteomic analysis (replicates of three parallel samples), according to the AIMS (Shanghai, China) (http://www.aimsmass.cn/index.jsp) to provide technical service. MS data were retrieved from UniPort database and analyzed using MaxQuant (version 1.6.1.0).

2.5. Biolayer interferometry

The affinity between PPD and wild-type PURA or its mutants were determined by BLI assay. Measurements were conducted at 30 °C refer to the previous description [21]. Detailed procedures are described in Supplementary Methods. The affinity of proteins and small molecules ($K_D = K_{off}/K_{on}$) was calculated using the final K_{on} and K_{off} rate constants.

2.6. Molecular docking

Discovery Studio 4.0 (Accelrys, San Diego, USA) was used to perform molecular docking calculations refer to previously described [23]. The Sketching protocol in DS 4.0 was used to construct and optimize the three-dimensional structure of the ligand. The structure of PURA predicted by AlphaFold was downloaded from protein databank (https://www.rcsb.org/) and preprocessed to remove water molecules and add hydrogen atoms for molecular docking. The detailed process of molecular docking refer to the previous description [6].

2.7. Site-directed mutagenesis

Amplification of the PURA open reading frame (ORF) was performed using primers (Supplementary Table 1) and cDNA from rat brain tissue as a template. Then, the PURA gene was cloned into the pET28a vector by homologous recombinase to obtain pET-PURA. Site-directed mutagenesis was performed using pET-PURA as a template to construct pET81, pET102, pET103, pET149, pET152, pET212, and pET238, which had mutations of lysine at position 81 to alanine (Mutant 1, Supplementary Figure 2), serine at position 102 to alanine (Mutant 2, Supplementary Figure 3), methionine at position 103 to alanine (Mutant 3, Supplementary Figure 4), arginine at position 149 to alanine (Mutant 4, Supplementary Figure 5), arginine at position 152 to alanine (Mutant 5, Supplementary Figure 6), glutamic acid at position 212 to alanine (Mutant 6, Supplementary Figure 7), and lysine at position 238 to alanine (Mutant 7, Supplementary Figure 8), respectively [12]. Detailed procedures are described in Supplementary Methods.

2.8. Protein expression and purification

The recombinant plasmids (pET-PURA, pET81, pET102, pET103, pET149, pET152, pET212, or pET238) were transformed into *Escherichia coli* BL21 (DE3) cells to express recombinant His-tagged PURA. The transformed BL21 (DE3) cells were cultured in Luria-Bertani medium containing 50 μg/mL kanamycin at 37 °C until the OD600 reached about 0.6. Then, the temperature was changed to 22 °C, and isopropyl-d-1-thiogalactopyranoside (IPTG) was used to induce *E. coli* cells for 18 h at a final concentration of 0.5 mM [12]. Detailed procedures of protein purification are provided in Supplementary Methods. Protein markers were used as reference proteins for molecular mass assessment, and protein purity was assessed by SDS-PAGE on a 10% polyacrylamide gel under denaturing conditions (Supplementary Figure 9).

2.9. Quantitative real-time PCR

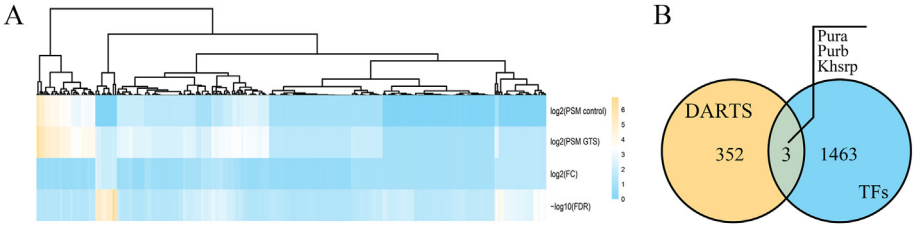
Twenty-four male adult rats were randomly divided into four groups (N = 6/group): (a) control group, (b) 2.5 mg/kg PPD group, (c) 5 mg/kg PPD group, and (d) 10 mg/kg PPD group. PPD was prepared at a concentration of 8 mg/mL by suspending PPD in physiological saline containing 0.02% dimethyl sulfoxide. The PPD suspension was used for intraperitoneal injection (i.p.) in the PPD group, whereas the control group received i.p. of the vehicle. Vehicle or PPD was i.p. daily for 1 week. Total RNA from hippocampal or cortex tissues (~50 mg) of rats were prepared by using Trizol reagent. HiScript II Q RT SuperMix kit was used to generate cDNA according to the manufacturer's instructions, and AceQ qPCR SYBR Green Master Mix was used to perform Real-time qPCR. The mRNA levels of *APP*, *Mbp*, *Fe65* (or *Apbb1*), *Plp1*, *nAchR* (or *Chrna*), and GAPDH in brain tissues were accessed. The sequences of the primers are shown in Supplementary Table 2.

2.10. Bioinformatics analysis

GO and KEGG analysis were performed by DAVID (https://david.ncifcrf.gov/). GO analysis mainly includes three types of literature sources or experimental verification evidence: biological process (BP), cellular component (CC) and molecular function (MF). KEGG analysis mainly uses existing knowledge of biochemical pathways and other types of molecular interactions for enrichment analysis. *P* value < 0.05 was taken as the criterion of statistical significance, and the results of GO and KEGG enrichment analysis were screened.

2.11. Statistical analysis

All statistical analyses in this study were performed using GraphPad Prism 8.0 software. The mean ± standard error of mean (SEM) was used to represent the results. Differences between treatment and control groups were analyzed using one-way



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IDs	Names	Descriptions	Fold Change	P. Value
1	PURA	Transcriptional activator protein Pur-alpha (Pura)	3	0.00132533
2	KSRP	Far upstream element-binding protein 2 (Khsrp)	1.666666667	0.043735123
3	PURB	Transcriptional activator protein Pur-beta (Purb)	1.5	0.041882455

Fig. 1. The protein targets of ginsenosides in brain tissue were identified by drug affinity response target stability (DARTS). (A) Heatmap of GTS-related protein targets was generated by mass spectrometry based DARTS technique. (B) Venn diagram of proteins at intersection with transcription factor library. Three overlapping transcription factors between DARTS-based protein targets and transcription factor database (TFs). (C) Specific information of the four transcription factors.

variance (ANOVA). $P < 0.05$ was considered statistically significant and $P < 0.01$ or $P < 0.001$were considered very significant.

3. Results

3.1. Preliminary screening of transcription targets of ginsenosides in brain tissues

DARTS allows direct comparison of protein profiles of GTS- and vehicle- treated tissue homogenates after protease digestion, thereby screening drug-stable proteins as potential targets. In the present study, DARTS was mainly accomplished by treating rat brain homogenates using GTS or pure water, followed by protease digestion. Subsequently, potential protein targets interacting with ginsenosides in brain tissues were identified using label-free LC-MS. As shown in Fig.1A, the total number of differential proteins in GTS treated groups was normalized to control group by LC-MS analysis and presented as a heatmap. Subsequently, the 355 differential proteins selected by DARTS were intercrossed with the transcription factor library (JASPA, https://jaspar.genereg.net), as shown in Fig.1B, and three transcription factors were intercrossed. Specific information on overlapping transcription factors is provided in Fig.1C.

3.2. PPD bound directly to PURA

In this study, the protein-protein interaction network of PURA was analyzed using the STRING online database (Fig. 2A), which has shown an interaction between PURA and PURB. The affinity between PURA and PURB was then measured using BLI, and the affinity (K_D) was 1.93 ± 0.11E-6 M (Fig. 2B). PURA was selected for

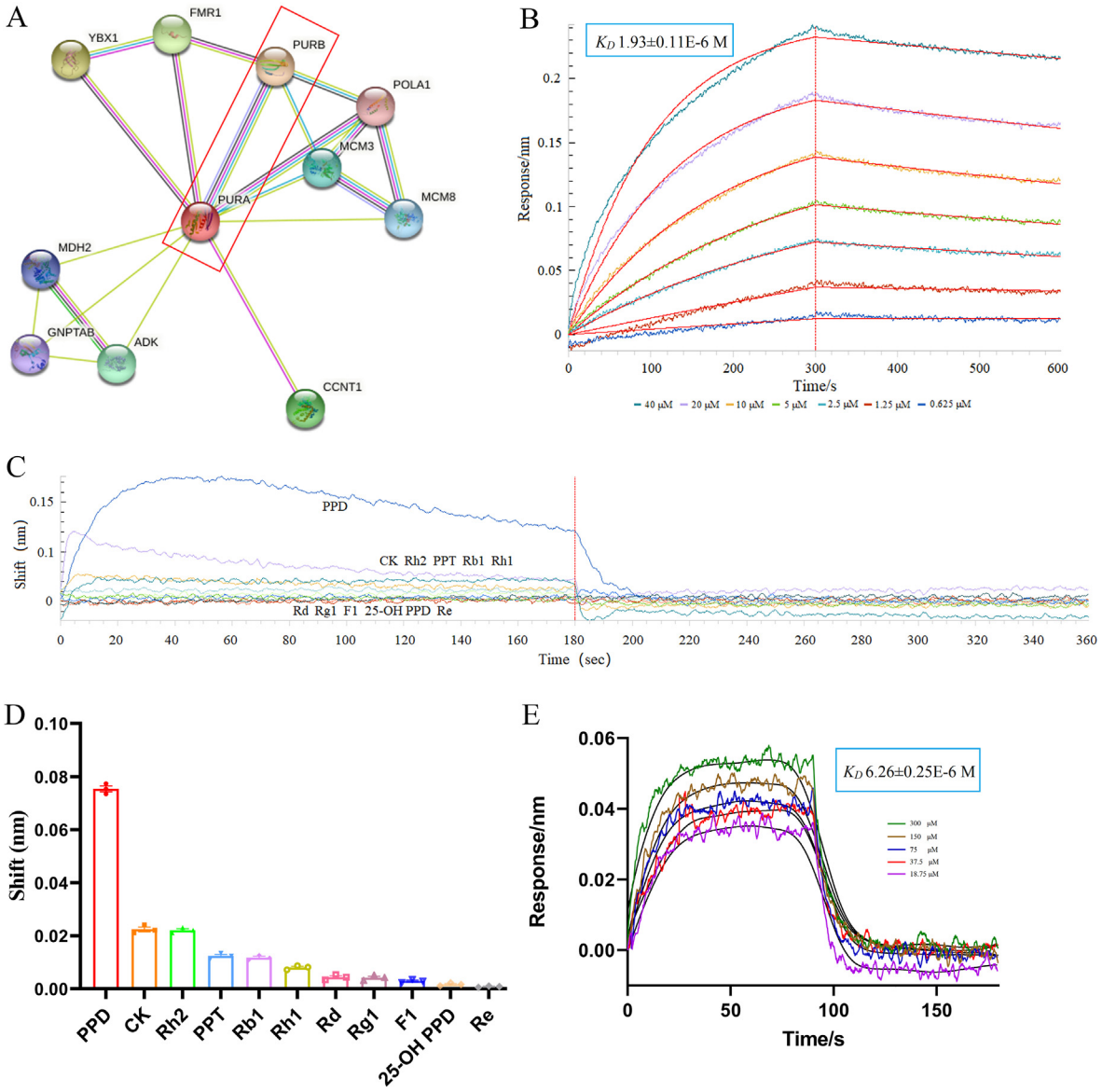


Fig. 2. Interaction of PURA with PURB or ginsenosides. (A) The STRING database analyzes the protein-protein interaction network of PURA, in which PURB is included. (B) BLI kinetic analysis was used to detect the affinity between PURA and PURB. The results were obtained from at least three experiments. (C) The representative direct interaction curves of the PURA to ginsenosides and aglycones were obtained through BLI analysis. (D) Statistical analysis plot of the direct interaction of PURA with ginsenosides and aglycones detected by BLI technology (mean ± SEM, n = 3). (E) BLI kinetic analysis was used to observe the affinity between PPD and PURA. PPD were set at 300, 150, 75, 37.5 and 18.75 μM, respectively. The results were obtained from triplicate experiments.

further study by comparing the Fold change and *p*-value of DARTS proteomics.

Direct binding was first determined by BLI, where PURA was immobilized on a biosensor using a His tag, and the wavelength shift was detected in real time after the addition of small molecules. The direct binding strength of PURA to Rb1,Re, Rh1, Rg1, F1, Rd, Rh2,CK, PPD, 25-OH PPD, and PPT at a concentration of 300 μM was shown in Fig. 2C and D. Among them, PPD showed the strongest direct binding activity with PURA and was used for further analysis. BLI Kinetic analysis showed that the affinity (K_D) of PPD for PURA was 6.26 ± 0.25E-6 M (Fig. 2E).

3.3. Molecular docking of PURA and PPD

Next, we performed molecular docking based on the structure of human PURA (IDENTIFIER: AF-Q00577-F1) predicted by AlphaFold using DS 4.0 software to explore the binding sites of PURA/PPD complex. Since there is currently no readily available active pocket for PURA, the From Receptor Cavities in the DS module was chosen to predict its active pocket. To find the best conformation that binds PURA better, the orientation, position, and conformation of the PPD were then sequentially adjusted using a search algorithm. Docking results showed that PPD interacted with the amino acid residues of the active site with hydrogen bonds (H bonds), which seemed to be more suitable for the active pocket (Fig. 3A). Since the amino acid sequence of human and rat PURA differs from one glycine in the glycine sequence of the anterior segment, there will be one amino acid difference in the corresponding amino acid site. Ser103 and Met104 in Fig. 3B and C actually corresponds to Ser102 and Met103 in the amino acid sequence of rat PURA. The 12-OH of PPD forms H-bonds with Ser102 (S102) and Met103 (M103) in the structure of rat PURA, and additional portions of PPD forms multiple hydrophobic interactions and a C–H bond with the active pocket of PURA.

3.4. S102 and M103 residues may be key sites for the interaction between PURA and PPD

Based on the key amino acids of the active site predicted by molecular docking (K81, S102, M103, R149, R152, E212 and K238, corresponding to K82, S103, M104, R150, R153, E213 and K239 in Fig. 3B and C), we then used site-directed mutagenesis of amino acids to obtain mutants of PURA. Firstly, the first mutant (K81A, Mutant 1) was obtained, in which the lysine at position 81 was changed to alanine. Then six other mutants (S102A, Mutant 2; M103A, Mutant 3; R149A, Mutant 4; R152A, Mutant 5; E212A, Mutant 6; K238A, Mutant 7) were generated by mutating serine at

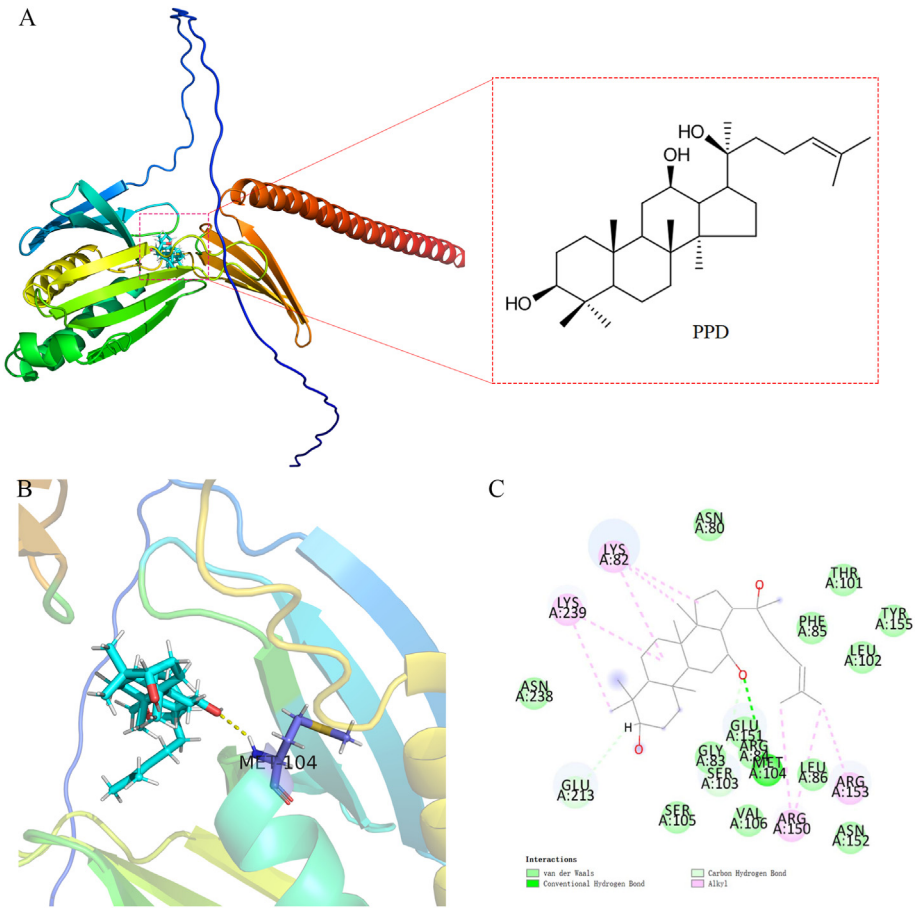
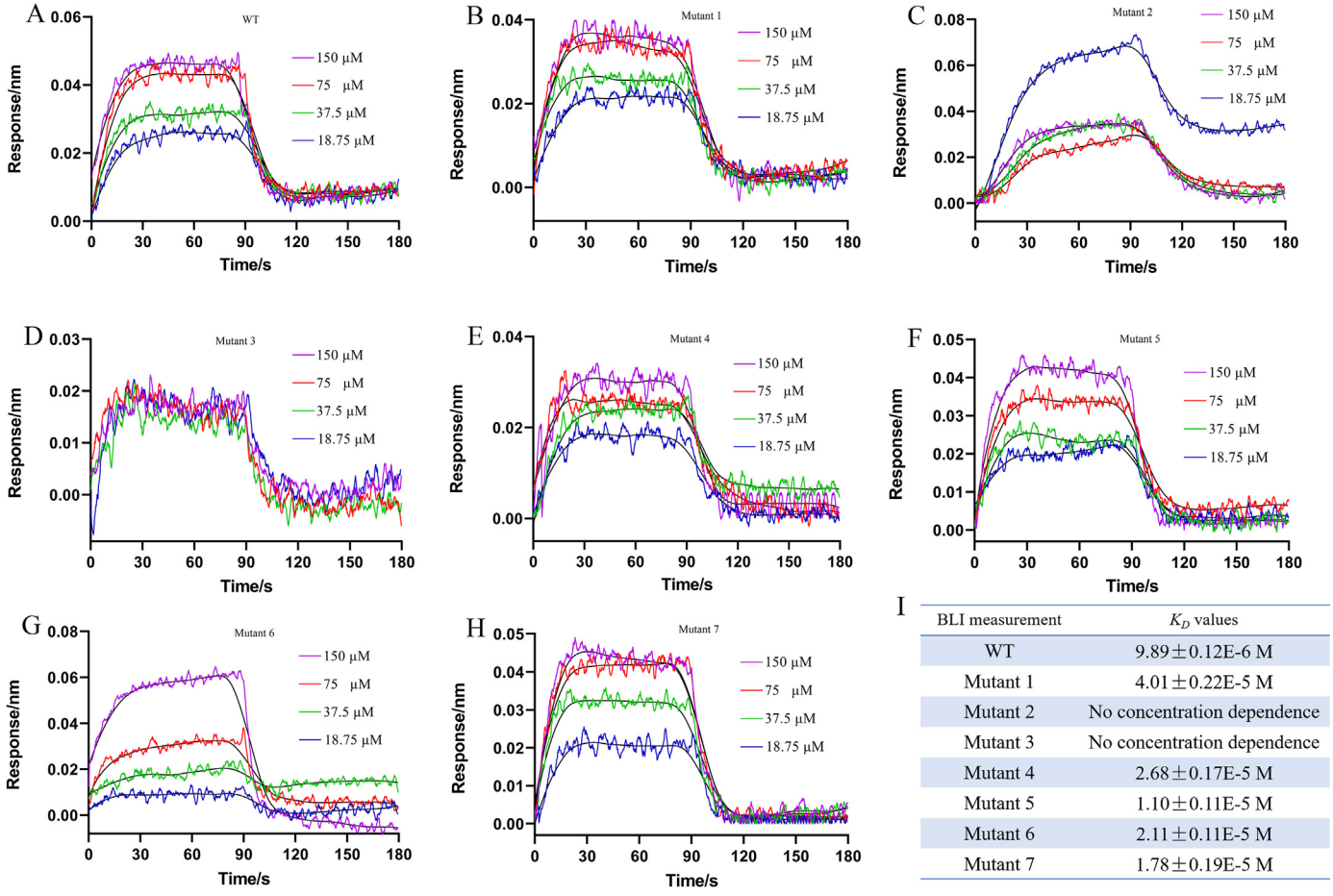


Fig. 3. Molecular docking studies on the PURA/PPD complex. Since the amino acid sequence of human and rat PURA differs from one glycine in the glycine sequence of the anterior segment, there will be one amino acid difference in the corresponding amino acid site. (A) Docking diagram of PPD with active sites in the Alphafold predicted PURA structure, where the 2D structure of PPD is located in the upper right corner of the panel. (B) Partial docking diagram of active sites in the structure of PPD and PURA, where PPD is represented by light blue sticks, key residues used for binding in PURA are represented by purple sticks, and key roles formed are represented by yellow dashed lines. (C) The 2D diagram of molecular docking between PURA and PPD.

position 102 to alanine, methionine at position 103 to alanine, arginine at position 149 to alanine, arginine at position 152 to alanine, glutamic acid at position 212 to alanine, and lysine at position 238 to alanine, respectively (Supplementary Figure 1). The affinity of PURA mutants and wild-type (WT) for PPD was examined by BLI kinetic analysis. The results showed that the K_D values of PPD and WT, Mutant 1, Mutant 2, Mutant 3, Mutant 4, Mutant 5, Mutant 6 and Mutant 7 were 9.89 ± 0.12E-6 M (Fig. 4A), 4.01 ± 0.22E-5 M (Fig. 4B), no concentration dependence (Fig. 4C), no concentration dependence (Figs. 4D), 2.68 ± 0.17E-5 M (Figs. 4E), 1.10 ± 0.11E-5 M (Figs. 4F), 2.11 ± 0.11E-5 M (Figs. 4G), and 1.78 ± 0.19E-5 M (Fig. 4H), respectively. As shown in Fig. 4I, although the affinity of WT for PPD was different from Mutant 1, Mutant 4, Mutant 5, Mutant 6 and Mutant 7, the difference was not particularly significant, but the affinity between PPD and Mutant 2 or Mutant 3 were not concentration dependent. These results suggested that PPD bound to the active sites of PURA. The mutation sites of Mutant 2 and Mutant 3 were adjacent to each other in the sequence, and mutation of either of them resulted in loss of affinity between PURA and PPD. The above data implied that the S102 and M103 residues were necessary for binding of PURA to PPD, and that other amino acid active sites might play a partial role in binding of PURA to PPD.

3.5. PPD binds PURA to upregulate the mRNA expression of PURA target genes in brain tissues

It is reported that PURA, as a transcription factor, had a variety of target genes to control transcription. In this study, target genes (*APP, Mbp, Fe65, Plp1, nAchR*) related to nervous system diseases in the literature were selected [24], and the transcriptional levels of target genes in the hippocampus and cortex of rats were measured by qRT-PCR to explore whether PPD could alter the transcription of PURA target genes by binding to PURA *in vivo*. As shown in Fig. 5A–E, after daily i.p. PPD administration for 1 week, 5 mg/kg of PPD significantly upregulated the mRNA expression levels of *APP*, *Fe65*, and *Mbp* in the hippocampus compared with the control group ($P < 0.001$), but had no significant effect on the mRNA expression of nAchR and Plp1. In addition, PPD at 10 mg/kg increased the mRNA expression of *APP*, *Mbp*, *nAchR*, and *Plp1* in the hippocampus ($P < 0.05$), but had no effect on the transcription level of *Fe65* (Fig. 5A–E). In the cortex, there was no statistically significant effect of PPD on *APP* transcript levels compared with controls (Fig. 5F). However, 5 mg/kg PPD greatly increased the mRNA expression of *Fe65*, *Mbp*, *nAchR* and *Plp1* in the cortex ($P < 0.001$, $P < 0.01$, and $P < 0.05$; Fig. 5F–J), and 10 mg/kg PPD increased the mRNA expression of *Mbp*, *nAchR* and *Plp1* ($P < 0.001$, $P < 0.01$, and



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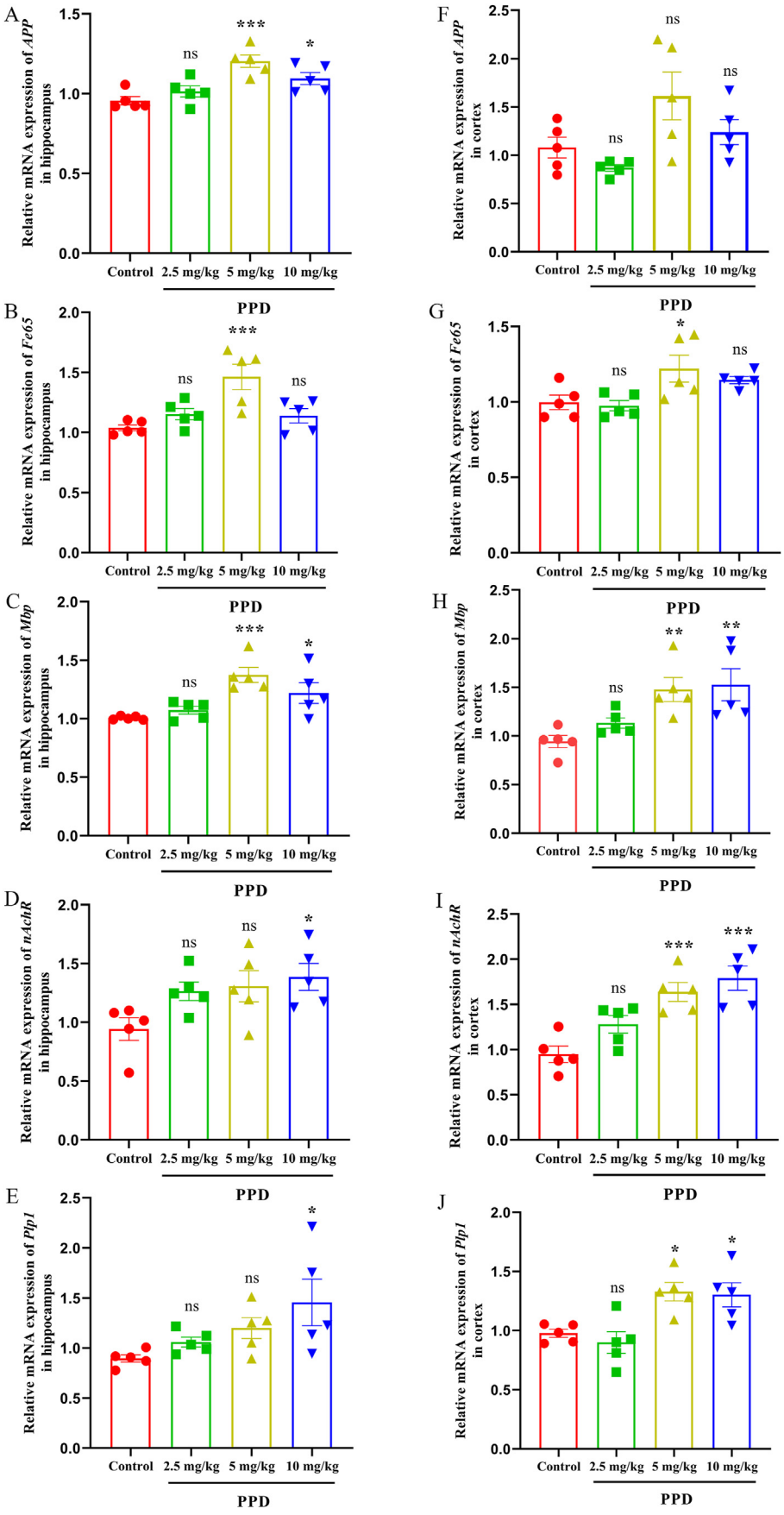
BLI measurement	K_D values
WT	9.89±0.12E-6 M
Mutant 1	4.01±0.22E-5 M
Mutant 2	No concentration dependence
Mutant 3	No concentration dependence
Mutant 4	2.68±0.17E-5 M
Mutant 5	1.10±0.11E-5 M
Mutant 6	2.11±0.11E-5 M
Mutant 7	1.78±0.19E-5 M

Fig. 4. S102 and M103 residues are required for PURA to have a high affinity for PPD. (A) The affinity between PPD and wild-type PURA (WT) was determined by BLI. The binding of PPD to (B) Mutant 1, (C) Mutant 2, (D) Mutant 3, (E) Mutant 4, (F) Mutant 5, (G) Mutant 6, and (H) Mutant 7 were also detected through BLI. The Ni-Nitrilotriacetic acid (Ni-NTA) biosensors were used to load PURA with His-tag, and then the sensors were incubated with different concentrations of PPD to form binding curves. The 90-s association curves were followed by 90-s dissociation curves. The results presented are representative of at least three experiments. (I) K_D values of WT and Mutants revealed that residues S102 and M103 are essential in the binding of PPD to the transcription factor PURA. Data are shown as mean ± SEM. (n = 3).


A
Relative mRNA expression of APP in hippocampus
ns
***
*
Control 2.5 mg/kg 5 mg/kg 10 mg/kg
PPD
F
Relative mRNA expression of APP in cortex
ns
ns
ns
B
Relative mRNA expression of Fe65 in hippocampus
ns
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ns
G
Relative mRNA expression of Fe65 in cortex
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C
Relative mRNA expression of Mbp in hippocampus
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E
Relative mRNA expression of Plp1 in hippocampus
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Relative mRNA expression of Plp1 in cortex
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$P < 0.05$; Fig. 5F–J). These results indicated that PPD interacts directly with PURA in brain tissue to affect transcription of PURA target genes.

3.6. Bioinformatics analysis

The potential biological characteristics of PURA, PURB, five target genes of PURA, and proteins directly interacted with them analyzed by STRING database were verified by GO and KEGG enrichment analysis of protein targets using DAVID service. A total of 39 proteins were used for analysis, and the top 10 highly enriched GO terms under BP, CC, and MF are shown in Fig. 6A–C. The results showed that multiple BP were associated with cognitive dysfunction, including beta-amyloid formation (GO: 0034205), amyloid precursor protein metabolic process (GO: 0042982), cellular response to beta-amyloid (GO: 01904646), amyloid precursor protein catabolic process (GO: 0042987), and learning or memory (GO: 0007611) (Fig. 6A). Synapse is the most important CC among the target proteins in CC classification (Fig. 6B). As shown in Fig. 6C, GO analysis revealed that these target proteins were associated with MF, and that beta-amyloid binding (GO: 0001540) was the most important MF for these target proteins. The KEGG analysis showed that these target proteins were associated with a variety of pathways, and the Alzheimer's disease-related pathway (hsa05010) was the most important pathway involved by these target proteins (Fig. 6D).

4. Discussion

Identification of protein targets of natural small molecules *in vivo* is crucial for studying their related functions. DARTS is a rapid and direct method for the discovery of protein targets of natural small-molecule drugs [25].The advantage of DARTS is that it does not require any chemical modification of small molecules for target recognition, so as to avoid tedious organic synthesis and loss of biological activity caused by changes in compound spatial structure, and can be used for screening and identification of protein targets for direct binding of small molecules [22]. Due to the rapid development of proteomics technology and instruments and the combination with mass spectrometry, various DARTS target screening combined with proteomics methods have emerged [26,27]. In this study, the potential targets of ginsenosides in brain tissue were screened by DARTS combined with label-free LC-MS. Highly purified GTS were used as ligands to ensure that protein targets of different ginsenosides could be discovered, and DARTS was used for target screening. By LC-MS analysis, we identified 355 differentially expressed proteins with fold changes greater than or equal to 1.5. Then it was intersected with the transcription factor library, and three transcription factors were intersected, including PURA, KSRP, and PURB. Based on the fold change and P.value of DARTS proteomics, PURA was selected for the following study.

PURA (purine-rich element binding protein alpha) is a member of PUR protein family, which plays a key role in DNA replication, transcription and RNA translation by binding to single - or double-stranded DNA or RNA [28]. As a pleiotropic transcription factor, PURA can directly bind to specific DNA or RNA sequences to form multimeric complexes, or bind to other transcription factors through protein-protein interactions, and indirectly promote or inhibit the binding of transcription factors to specific gene promoters to exert transcriptional activation or repression. In this study, the direct interaction of PURA with ginsenosides (Rd, Rb1, Re and Rg1) and their metabolites (Rh2, Rh1, F1, PPT, CK, PPD and 25-OH PPD) was detected by BLI (Fig. 2C and D). Next, we selected the PPD with the strongest direct effect to investigate the affinity between it and the transcription factor PURA. BLI kinetic analysis showed an affinity between PPD and PURA (Fig. 2E). Molecular docking results showed that PPD could sequentially attach to the active pocket of PURA predicted by DS 4.0 (Fig. 3A). Moreover, BLI kinetic analysis indicated that mutation of major amino acids (K81, R149, R152, E212, and K238) in the active site to form hydrogen bond or hydrophobic interaction did not significantly affect the interaction between PPD and PURA, but mutation of critical amino acids (S102 and M103) for hydrogen bond formation resulted in loss of PPD binding ability to PURA (Fig. 4).

PURA was originally discovered in HeLa cells and was thought to function as an SSDNA-binding factor. It was subsequently isolated from mouse brain lysates and found to bind to the promoter region of myelin basic protein (MBP) [24]. PURA acts as a transcription factor that regulates the transcription levels of its target genes, and it is frequently shown in high-throughput analysis of neuronal tissue interaction networks. Previous studies have shown that PURA plays a key role in brain development, neurological function and disease [29]. For example, heterozygous loss of PURA is associated with memory deficits in mice [30]. Khalili et al. showed that knockout of heterozygous PURA in mice, while surviving, exhibited seizure-like disorders [31]. Moreover, de novo mutations in PURA have been demonstrated to be involved in hypotonia and developmental delay [32]. In addition, it has been suggested that PURA may play a neuroprotective role in neurodegenerative diseases. Xu et al. [33] found that PURA, as the RNA-binding protein, was implicated in the pathogenesis of frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), and Shi et al. [34] analyzed the role of PURA in the pathogenesis of Alzheimer's disease by ChIP-seq and RNA-seq, and found that PURA may play a regulatory role in some AD-related genes through direct and indirect ways in the pathogenesis of AD.

Given the identification of the transcription factor target PURA from brain tissue in this study and the key role of PURA in the nervous system, we next detected the effect of PPD on the transcriptional levels of PURA target genes in brain tissue *in vivo*. In addition, due to the relatively low bioavailability of PPD when administered orally, i.p. was primarily used in the *in vivo* experiments. Five target genes of PURA in brain tissues were identified from the literature [24], including *APP*, *Mbp*, *Fe65*, *Plp1* and *nAchR*. The results of qRT-PCR indicated that PPD could observably alter the transcriptional levels of PURA target genes in vivo (Fig. 5). According to studies *in vivo*, daily i.p. of 5 mg/kg or 10 mg/kg PPD increased the transcriptional levels of most target genes, such as *Mbp*, *Fe65*, *Plp1* and *nAchR* in cortex, and *APP*, *Mbp*, *Fe65*, *Plp1* and *nAchR* in hippocampus, but 2.5 mg/kg PPD had no statistically significant effect on PURA target genes. Interestingly, PPD increased the transcriptional levels of PURA target genes *nAchR* and *Plp1* in the hippocampus and *nAchR* and *Mbp* in the cortex in a dose-dependent manner.

Bioinformatics analysis was used to analyze the potential biological features of PURA, PURB, *APP*, *Mbp*, *Fe65*, *Plp1*, *nAchR* and 32 proteins directly interacted with them. Several enriched BP were identified by GO-based bioinformatics analysis, including beta-amyloid formation, amyloid precursor protein metabolic process, cellular response to beta-amyloid, amyloid precursor protein

Fig. 5. Effects of PPD on transcription levels of PURA target genes. Rats in the experimental group were intraperitoneally injected with PPD of 2.5 mg/kg, 5 mg/kg and 10 mg/kg, respectively, while rats in the control group were intraperitoneally injected with vehicle, daily injection for 1 week. After treatment for 1 week, the transcription levels of PURA target genes *APP*, *Fe65*, *Mbp*, *Plp1*, and *nAchR* in hippocampus (A-E) and prefrontal cortex (F-J) were detected by qRT-PCR (mean ± SEM, n = 5).
$^{***}P < 0.001$ vs. the control group, $^{**}P < 0.01$ vs. the control group, $^{*}P < 0.05$ vs. the control group, and ^{ns} no statistical significance vs. the control group.

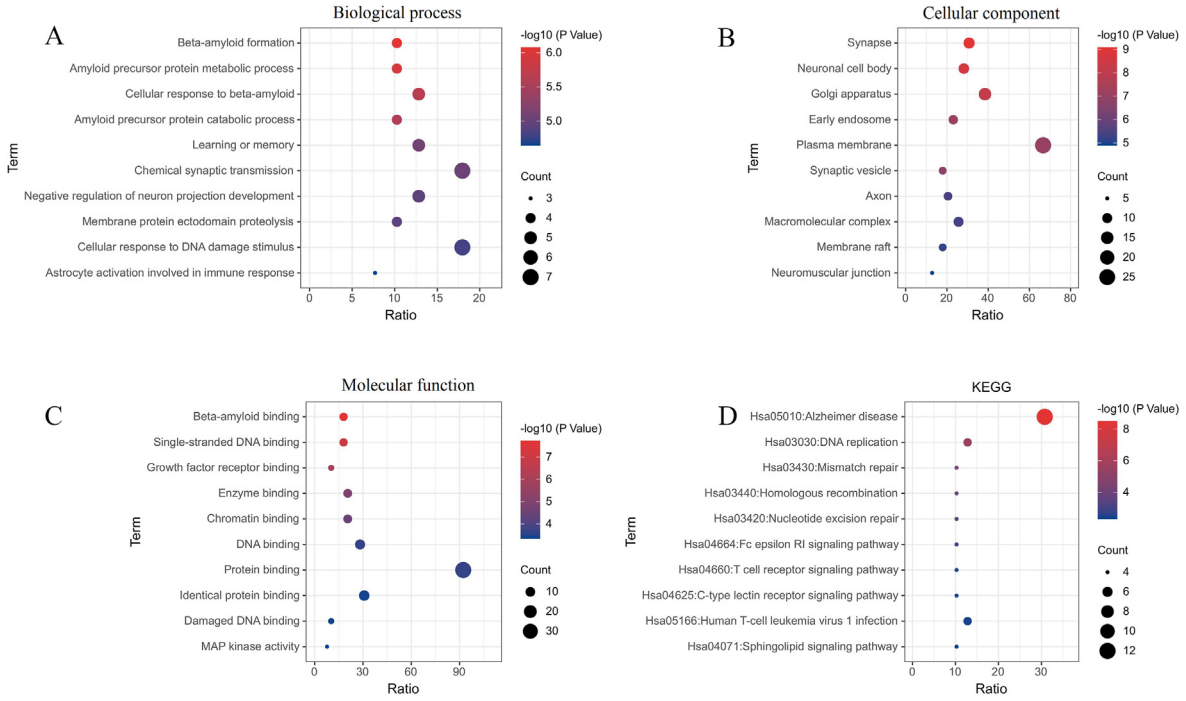

Fig. 6. Bioinformatics analysis the potential biological features of target proteins involved in PURA, PURB, five target genes of PURA, and proteins directly interacted with them analyzed by STRING database. GO enrichment analysis (A) Top 10 significantly enriched biological process. (B) Top 10 significantly enriched cellular component. (C) Top 10 significantly enriched molecular function. (D) Top 10 pathways were significantly enriched by KEGG analysis.

catabolic process, and learning or memory (Fig. 6A–C). Combined with the results of our KEGG analysis (Fig. 6D), we found that BP and pathway associated with cognitive dysfunction were particularly abundant, suggesting that PPD may play a key role in the improvement of cognitive dysfunction by targeting PURA. In fact, studies have shown that these target genes of PURA play key roles in cognitive dysfunction. For example, FE65 (or APBB1) is a bridging protein that binds to amyloid precursor protein (APP) and is therefore involved in the pathogenesis of AD [35]. As a kind of pentameric ligand-gated ion channel, Nicotinic acetylcholine receptors (nAChRs) are involved in the regulation of some basic brain functions such as memory, learning and attention [36]. Moreover, the mutation of *Plp1* gene resulted in myelination failure and neurological dysfunction in X-chromosome linked white matter dystrophy Pelizaeus-Merzbacher disease (PMD) [37]. In addition, it also regulates neurodevelopment and synaptic function, such as learning and memory deficits reported in FE65 knockout mice, which are associated with synaptic changes [38]. Given the important role of these genes or proteins in cognitive dysfunction, PPD may improve cognitive dysfunction by binding with PURA to alter the transcription levels of these target genes. However, it remains to be further studied how PPD upregulates the mRNA expression of these target genes by binding to PURA.

5. Conclusion

In this research, we demonstrated for the first time that PURA may be a transcription target of ginsenosides in brain tissue. Ginsenoside metabolites could bind to PURA directly, and PPD had the highest affinity. PPD could increase the transcriptional levels of PURA target genes by binding to PURA. Bioinformatics analysis revealed that PPD plays a key role in the improvement of cognitive dysfunction targeting PURA. Our results provide a basis for the mechanistic study of ginseng bioactivities in the nervous system and the development of small molecule compounds targeting PURA.

Contributors

Yunan Zhao and Feiyan Chen designed the project. Yunan Zhao designed and conducted DARTS. Feiyan Chen performed intermolecular interaction detections, mutagenesis, qRT-PCR and Bioinformatics analysis. Wenjing Zhang designed and conducted molecular docking. Shuyi Xu and Hantao Zhang performed animal experiments and collected tissue samples. Cuihua Chen and Lin Chen assisted in the data analysis. Feiyan Chen, Zhu Zhu and Yunan Zhao wrote the manuscript. All authors discussed and commented on the manuscript.

Declaration of competing interest

The authors have declared that there is no conflict of interest.

Acknowledgements

The study was financially supported by National Natural Science Foundation of China (No.82204647, 82003970), Supporting project of National Natural Youth Foundation of Nanjing University of Chinese Medicine (XPT82204647).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2023.04.007.

References

[1] Lambert SA, Jolma A, Campitelli LF, Das PK, Yin Y, Albu M, et al. The human transcription factors. Cell 2018;172:650–65.

[2] Goswami S, Kareem O, Goyal RK, Mumtaz SM, Tonk RK, Gupta R, et al. Role of forkhead transcription factors of the O class (FoxO) in development and progression of Alzheimer's disease. CNS & Neurological Disorders Drug Targets 2020;19:709–21.
[3] Vargas DM, De Bastiani MA, Zimmer ER, Klamt F. Alzheimer's disease master regulators analysis: search for potential molecular targets and drug repositioning candidates. Alzheimer's Research & Therapy 2018;10:59.
[4] Dard RF, Dahan L, Rampon C. Targeting hippocampal adult neurogenesis using transcription factors to reduce Alzheimer's disease-associated memory impairments. Hippocampus 2019;29:579–86.
[5] Català-Solsona J, Miñano-Molina AJ, Rodríguez-Álvarez J. Nr4a2 transcription factor in hippocampal synaptic plasticity, memory and cognitive dysfunction: a perspective review. Frontiers in Molecular Neuroscience 2021;14:786226.
[6] Chen F, Li C, Cao H, Zhang H, Lu C, Li R, et al. Identification of adenylate kinase 5 as a protein target of ginsenosides in brain tissues using mass spectrometry-based drug affinity responsive target stability (DARTS) and cellular thermal shift assay (CETSA) techniques. Journal of Agricultural and Food Chemistry 2022;70:2741–51.
[7] Pan W, Xue B, Yang C, Miao L, Zhou L, Chen Q, et al. Biopharmaceutical characters and bioavailability improving strategies of ginsenosides. Fitoterapia 2018;129:272–82.
[8] Xu ZL, Chen G, Liu X, Xie D, Zhang J, Ying Y. Effects of ginsenosides on memory impairment in propofol-anesthetized rats. Bioengineered 2022;13:617–23.
[9] Wang Q, Sun LH, Jia W, Liu XM, Dang HX, Mai WL, et al. Comparison of ginsenosides Rg1 and Rb1 for their effects on improving scopolamine-induced learning and memory impairment in mice. Phytotherapy Research : PTR 2010;24:1748–54.
[10] Yang R, Jiang X, He X, Liang D, Sun S, Zhou G. Ginsenoside Rb1 improves cognitive impairment induced by insulin resistance through cdk5/p35-NMDAR-IDE pathway. BioMed Research International 2020;2020:3905719.
[11] Cui L, Wu SQ, Zhao CA, Yin CR. Microbial conversion of major ginsenosides in ginseng total saponins by Platycodon grandiflorum endophytes. Journal of Ginseng Research 2016;40:366–74.
[12] Chen F, Chen L, Liang W, Zhang Z, Li J, Zheng W, et al. Identification and confirmation of 14-3-3 ζ as a novel target of ginsenosides in brain tissues. Journal of Ginseng Research 2021;45:465–72.
[13] Musende AG, Eberding A, Wood CA, Adomat H, Fazli L, Hurtado-Coll A, et al. A novel oral dosage formulation of the ginsenoside aglycone protopanaxadiol exhibits therapeutic activity against a hormone-insensitive model of prostate cancer. Anti-cancer Drugs 2012;23:543–52.
[14] Chen C, Wang L, Cao F, Miao X, Chen T, Chang Q, et al. Formulation of 20(S)-protopanaxadiol nanocrystals to improve oral bioavailability and brain delivery. International Journal of Pharmaceutics 2016;497:239–47.
[15] Lu C, Dong L, Lv J, Wang Y, Fan B, Wang F, et al. 20(S)-protopanaxadiol (PPD) alleviates scopolamine-induced memory impairment via regulation of cholinergic and antioxidant systems, and expression of Egr-1, c-Fos and c-Jun in mice. Chemico-biological Interactions 2018;279:64–72.
[16] Lu C, Lv J, Dong L, Jiang N, Wang Y, Fan B, et al. The protective effect of 20(S)-protopanaxadiol (PPD) against chronic sleep deprivation (CSD)-induced memory impairments in mice. Brain Research Bulletin 2018;137:249–56.
[17] Ren YS, Li HL, Piao XH, Yang ZY, Wang SM, Ge YW. Drug affinity responsive target stability (DARTS) accelerated small molecules target discovery: principles and application. Biochemical Pharmacology 2021;194:114798.
[18] Lomenick B, Hao R, Jonai N, Chin RM, Aghajan M, Warburton S, et al. Target identification using drug affinity responsive target stability (DARTS). Proceedings of the National Academy of Sciences of the United States of America 2009;106:21984–9.
[19] Friman T. Mass spectrometry-based Cellular Thermal Shift Assay (CETSA®) for target deconvolution in phenotypic drug discovery. Bioorganic & Medicinal Chemistry 2020;28:115174.
[20] Kanai Y, Dohmae N, Hirokawa N. Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. Neuron 2004;43:513–25.
[21] Chen F, Zhu K, Chen L, Ouyang L, Chen C, Gu L, et al. Protein target identification of ginsenosides in skeletal muscle tissues: discovery of natural small-molecule activators of muscle-type creatine kinase. Journal of Ginseng Research 2020;44:461–74.
[22] Pai MY, Lomenick B, Hwang H, Schiestl R, McBride W, Loo JA, et al. Drug affinity responsive target stability (DARTS) for small-molecule target identification. Methods in Molecular Biology (Clifton, NJ) 2015;1263:287–98.
[23] Lu C, Fan L, Zhang PF, Tao WW, Yang CB, Shang EX, et al. A novel P38α MAPK activator Bruceine A exhibits potent anti-pancreatic cancer activity. Computational and Structural Biotechnology Journal 2021;19:3437–50.
[24] Molitor L, Bacher S, Burczyk S, Niessing D. The molecular function of PURA and its implications in neurological diseases. Frontiers in Genetics 2021;12:638217.
[25] Lomenick B, Jung G, Wohlschlegel JA, Huang J. Target identification using drug affinity responsive target stability (DARTS). Current Protocols in Chemical Biology 2011;3:163–80.
[26] Yang Z, Kuboyama T, Tohda C. A systematic strategy for discovering a therapeutic drug for Alzheimer's disease and its target molecule. Frontiers in Pharmacology 2017;8:340.
[27] Sun J, Prabhu N, Tang J, Yang F, Jia L, Guo J, et al. Recent advances in proteome-wide label-free target deconvolution for bioactive small molecules. Medicinal Research Reviews 2021;41:2893–926.
[28] Johnson EM, Daniel DC, Gordon J. The pur protein family: genetic and structural features in development and disease. Journal of Cellular Physiology 2013;228:930–7.
[29] Daniel DC, Johnson EM. PURA, the gene encoding Pur-alpha, member of an ancient nucleic acid-binding protein family with mammalian neurological functions. Gene 2018;643:133–43.
[30] Barbe MF, Krueger JJ, Loomis R, Otte J, Gordon J. Memory deficits, gait ataxia and neuronal loss in the hippocampus and cerebellum in mice that are heterozygous for Pur-alpha. Neuroscience 2016;337:177–90.
[31] Khalili K, Del Valle L, Muralidharan V, Gault WJ, Darbinian N, Otte J, et al. Puralpha is essential for postnatal brain development and developmentally coupled cellular proliferation as revealed by genetic inactivation in the mouse. Molecular and Cellular Biology 2003;23:6857–75.
[32] Tanaka AJ, Bai R, Cho MT, Anyane-Yeboa K, Ahimaz P, Wilson AL, et al. De novo mutations in PURA are associated with hypotonia and developmental delay. Cold Spring Harbor Molecular Case Studies 2015;1:a000356.
[33] Xu Z, Poidevin M, Li X, Li Y, Shu L, Nelson DL, et al. Expanded GGGGCC repeat RNA associated with amyotrophic lateral sclerosis and frontotemporal dementia causes neurodegeneration. Proceedings of the National Academy of Sciences of the United States of America 2013;110:7778–83.
[34] Shi X, Ren S, Zhang B, Guo S, He W, Yuan C, et al. Analysis of the role of Purα in the pathogenesis of Alzheimer's disease based on RNA-seq and ChIP-seq. Scientific Reports 2021;11:12178.
[35] Cheung HN, Dunbar C, Mórotz GM, Cheng WH, Chan HY, Miller CC, et al. FE65 interacts with ADP-ribosylation factor 6 to promote neurite outgrowth. official publication of the Federation of American Societies for Experimental Biology FASEB Journal 2014;28:337–49.
[36] Borroni V, Barrantes FJ. Homomeric and heteromeric α7 nicotinic acetylcholine receptors in health and some central nervous system diseases. Membranes 2021:11.
[37] Elitt MS, Barbar L, Shick HE, Powers BE, Maeno-Hikichi Y, Madhavan M, et al. Suppression of proteolipid protein rescues Pelizaeus-Merzbacher disease. Nature 2020;585:397–403.
[38] Wang Y, Zhang M, Moon C, Hu Q, Wang B, Martin G, et al. The APP-interacting protein FE65 is required for hippocampus-dependent learning and long-term potentiation. Learning & Memory (Cold Spring Harbor, NY) 2009;16:537–44.