New insights into the brain protein metabolism of Gastrodia elata-treated rats by quantitative proteomics

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ABSTRACT

Gastrodia elata (tianma) is a traditional Chinese herbal medicine (TCM) often used for the treatment of cerebrovascular diseases. In this study, we investigated the effects of tianma on the brain protein metabolism by quantitative proteomics to gain evidence for a direct relationship between tianma treatment and brain functions. One-year-old rats were treated with tianma (~2.5 g/kg/day) for 3 months and the brain tissue proteome was analyzed by using the iTRAQ (isobaric tag for relative and absolute quantification) technology. According to our results, the long-term treatment with tianma could modulate the brain protein metabolism at the proteome level by down-regulating the expressions of various proteins, such as Gnao1 and Dctn2, which are related to neuronal growth cone control and synaptic activities. In addition, tianma treatment also induced the up-regulation of molecular chaperons and proteins related to the misfolded protein response, like Anxa5, and also other proteins involved in Huntington’s disease (HD) (e.g. Pacsin1 and Arf3). Concluding, tianma could eventually contribute to activities related to synaptic plasticity and neurorestorative processes and thus might be a novel candidate agent for the treatment of neurodegenerative diseases by regulating the brain proteome.

1. Introduction

Over the past two decades research, development and usage of natural herbal products for therapeutic applications have attracted much attention for the treatment of diseases and disorders that are little responsive to current western medicine [1–5]. Tianma is one herbal medicine derived from the tuber of Gastrodia elata Blume that has been used in China and other oriental countries for centuries to treat various nervous and cerebrovascular diseases, such as hemiplegia, stroke, headaches, vertigo, convulsion and epilepsy. Numerous reports have addressed that tianma also could exert therapeutic effects against neurodegenerative diseases (NDs), such as HD, Parkinson’s disease (PD) and Alzheimer’s disease (AD) [5–8]. ND is the most common cause of dementia among elderly people and is characterized by the progressive loss of structure and function of neurons due to the death of neurons caused by abnormal protein aggregation, which is called amyloid deposits in histochemical stainings [9]. Tianma contains various bioactive components [10–14], which could cross the blood–brain...
barrier [15] to improve neuronal cell viability by inhibiting apoptosis [16,17] and to protect neuronal cells damaged by transient global brain ischemia [18]. It has also been reported that tianma could eventually prevent AD- and PD-related cell death [17,19–22]. However, the underlying molecular and cellular mechanisms are barely known.

Currently, much progress in genomic and proteomic technologies offer ample opportunities to study the entire genome or proteome of various cell and tissue samples in a single experiment. The two-dimensional (2D) liquid chromatography coupled with tandem mass spectrometry (2D-LC-MS/MS)-based multidimensional protein identification technology [23] combined with multiplex isobaric tag for relative and absolute quantification (iTRAQ) [24] provides a sensitive technical approach for cutting-edge quantitative proteomics profiling [25–27]. In order to unravel the potential mechanisms on how tianma regulates brain functions and neuroregenerative processes in the brain, we investigated the effect of tianma on brain proteome changes using iTRAQ after long-term treatment of one-year-old rats with tianma. The selective iTRAQ-detected changed proteins were further verified at the protein level by using western blot analyses (Fig. 1). Our experimental results showed for the first time that tianma could regulate the cellular brain protein metabolism.

2. Materials and methods

2.1. Reagents

Unless indicated, all reagents used for biochemical methods were purchased from Sigma-Aldrich (St. Louis, MO, USA). Materials and reagents for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). The iTRAQ reagent multi-plex kit, containing the iTRAQ reagents, was bought commercially (Applied Biosystems, Foster City, CA, USA).

2.2. Antibodies

Anti-Anxa5 (annexin V, 1:2500, rabbit polyclonal; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-Arf3 (ADP-ribosylation factor 3, 1:2500, goat polyclonal; Santa Cruz), anti-Gapdh (glyceraldehyde-3-phosphate dehydrogenase, 1:2500, goat polyclonal; Santa Cruz), anti-Dctn2 (dynactin 2, 1:1500, goat polyclonal; Santa Cruz), anti-Ncam (neural cell adhesion molecule, 1:2500, rabbit polyclonal; Cell Signalling Technology Inc., Danvers, MA, USA), anti-Pacsin1 (protein kinase C and casein kinase substrate in neurons protein 1, 1:2500, rabbit polyclonal; Santa Cruz) and anti-Sncb (synuclein-beta, 1:2500, rabbit polyclonal; Abcam, Cambridge, UK).

2.3. Animal material

Experimental procedures, including the killing of animals, were in accordance with the International Guiding Principles for Animal Research (WHO) and were approved by the local Institutional Animal Care & Use Committee (NTU-IACUC). One-year-old male Wistar Kyoto rats (~250 g) were obtained from the laboratory animal center (National University of Singapore) and randomly assigned to control and tianma-treated groups (12 each, Fig. 1). According to previous reports and our own recent studies, the average daily dose of tianma per rat was 2.5 g/kg body weight [22,28–31]. They were fed a normal chow (Funabashi SP, Japan) and tap water was given freely. Room temperature (RT) was kept at 21 ± 2 °C, with 60% humidity, and a 12 h light/dark cycle. Tianma-feeding was done orally (intragastric administration) with a blunt needle syringe by dispensing the tianma solution drop by drop for the period of 3 months. Control rats were treated with the same volume of the solvent only. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.4. Herb preparation

The rhizome of G. elata (tianma), grown under standardized conditions [32], was collected from Zhaotong City, China and provided by Dr. Jun Zhou (Kunming Institute of Botany, Chinese Academy of Science, Yunnan, P.R. China). The species was identified and chemically analyzed as reported previously [22,33]. A voucher specimen (0249742) was deposited in the herbarium of the Kunming Institute of Botany, (Chinese Academy of Science, Yunnan, P.R. China). In this study, whole dried tubers of the tianma were hammered into smaller pieces and subsequently ground to fine powder. 7.5 g of tianma powder was mixed with 100 ml sterilized Milli-Q water and boiled for 1 h at 100 °C. The solution was centrifuged at 5000×g for 10 min at RT. The supernatant was filtered with a Whatman filter paper-1 (GE Healthcare, Chalfont St Giles, UK), yielding approximately 85 ml. The tianma solution was concentrated at 60 °C under vacuum and the final volume was reduced to 10 ml for further applications.

2.5. Brain-tissue-specific protein expression analysis

For the brain-tissue-specific protein expression analyses, brain tissues were isolated from tianma-treated and control rats. Briefly, brains were excised from the rats gently after dissection, immediately immersed into liquid nitrogen, and then powdered using a mortar and pestle. Upon the addition of lysis buffer (2% SDS, 0.5 M Triethyl ammonium bicarbonate buffer (TEAB), 1 Complete™ protease inhibitor cocktail tablet (Roche, Mannheim, Germany) and 1 PhosSTOP phosphatase inhibitor cocktail tablet (Roche)), the samples were vortexed for 1 min and incubated on ice for an additional 45 min prior to homogenization (sonication parameters: amplitude, 23%; pulse: 5 s/s 5 s for 5 min) using a Vibra Cell high intensity ultrasonic processor (Jencon Scientific Ltd, Leighton Buzzard, Bedfordshire, UK). After centrifugation (20,000×g/4 °C/30 min), supernatant was collected and stored at −80 °C until further use. The protein concentration was quantified by a ‘2-D Quant’ kit (Amersham, Piscataway, NJ, USA) according to the manufacturer’s protocol.

2.6. ITRAQ Protocol [26, 27]

2.6.1. Sample preparation–acetone precipitation

Each sample condition had 600 μg of total protein lysate transferred to a new tube. Six volumes of 100% –20 °C-chilled acetone were added to each tube and vortexed thoroughly at
Following tianma (+T) treatment (batches BI (6 rats) and BII (6 rats); control (C)=BI (6 rats) and BII (6 rats)) and brain tissue lysis, protein extracts were acetone precipitated and quantified. These were then run in SDS-PAGE and subsequently digested. The quantitative proteomics analyses of each peptide solution from the different samples were performed by labeling with multi-plex isobaric tags (114, 115, 116 and 117) for relative and absolute quantification (iTRAQ) reagent followed by Electrostatic Repulsion–Hydrophilic Interaction Chromatography (ERLIC)-based fractionation, and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)-based multidimensional protein identification technology. The obtained data was analyzed using ProteinPilot software and validated by quantitative western blots. Finally, proteins were functionally classified into various subgroups.
regular intervals. The tubes were incubated overnight at -20 °C and the following day, vortexed and centrifuged at 16,000 × g for 30 min to pellet down all proteins. The supernatant was discarded and the pellets were disturbed and washed in 500 μl of 90% –20 °C-chilled acetone. Subsequently, the tubes were centrifuged at 16,000 × g for 20 min and the supernatant discarded. The washed pellets were allowed to air-dry at RT for 15 min, then dissolved in 100 μl of 200 mM TEAB (0.5 M Triethylammonium bicarbonate buffer), and 2% SDS and then incubated at 50 °C for 5–10 min with simple agitation using a thermomixer (Eppendorf, Hamburg, Germany). The tubes were centrifuged at 16,000 × g for 30 min. The supernatant was collected and protein concentration was re-quantified using the 2-D Quant kit.

2.6.2. SDS-PAGE and in-gel digestion
Two hundred μg of acetone-precipitated proteins was mixed with loading dye, denatured for 10 min in a thermo bath (Fine PCR, Seoul, Korea) and resolved up to 60% by SDS-PAGE. The gels were washed twice with autoclaved Milli-Q Water (MQW) for 5 min each and fixed overnight on a SH30L reciprocating shaker (Fine PCR) in 50% methanol and 10% Acetic Acid (AcOH). The gels were then washed with MQW thrice for 15 min each followed by in-gel digestion in a laminar flow hood (Gelman, Singapore). The gels were diced into 1–2 mm pieces and transferred into tubes. 5 ml of 25 mM TEAB in 50% Acetonitrile (ACN) buffer was added to the tubes, vortexed and left at RT for 10 min after which buffer was discarded and the step repeated four times. Finally, 80% ACN in 20 mM TEAB was added, vortexed, and the tubes were left at RT for 10 min. The supernatant was discarded and the sample tubes were left to air-dry for 30 min.

2.6.3. Reduction, alkylation, trypsin digestion and extraction
Stock solutions of 200 mM tris(2-carboxyethyl)phosphine (TCEP) in HPLC water (J.T. Baker, Mallinckrodt, Inc., Phillipsburg, NJ, USA) and 200 mM S-methyl methanethiosulfonate (MMTS) in isopropanol were prepared. 5 mM of TCEP in 25 mM TEAB buffer was added to the dried gel pieces, vortexed and briefly spun before being incubated at 65 °C for 1 h to allow a reduction reaction to take place. Following this, 10 mM MMTS in 25 mM TEAB buffer (tube was covered with aluminum foil) was added to gel pieces, vortexed, and briefly spun. The alkylation reaction was then allowed to proceed for 45 min in the dark at RT. The supernatant was removed and discarded. The gel pieces were again washed with 25 mM TEAB in 50% ACN buffer as described above. The gel was dehydrated by 100% ACN. Finally, the tubes were air-dried for 30 min. First, trypsin (4 μg of trypsin in 25 mM TEAB) was added to each set of the gel pieces and incubated at 4 °C for 15 min for proper rehydration. The tubes were spun shortly and incubated in a 37 °C incubator overnight.

Subsequently, the tubes were spun briefly and the aqueous extract of the digested solution was collected. To the remaining gel pieces, 50% ACN and 1% AcOH were added, vortexed, and incubated in a water bath sonicator for 30 min. The supernatant was transferred and combined to the main sample tube. The extraction step was repeated 5 times. The trypsin digested peptides were pooled and dried completely in the SpeedVac (Concentrator 5301, Eppendorf) at 30 °C and stored at –20 °C.

2.6.4. Labeling of peptides with iTRAQ tags (4 plex)
Each iTRAQ reagent tube (tags —114, 115, 116, 117) had 70 μl of 100% ethanol added and vortexed thoroughly. The dried peptides were dissolved in 30 μl of 500 mM TEAB (dissolution buffer). Each iTRAQ tag was transferred to the respective peptide tubes and the tubes were incubated at RT for 2 h with gentle shaking (thermomixer). All samples were then combined and kept in the SpeedVac at 30 °C to dry completely.

2.6.5. Desalting
The dried peptide samples were reconstituted in 500 μl of 0.1% formic acid (FA) and kept in the water bath sonicator for 5 min. A 50 mg C18 cartridge (Sep-Pak® Vac C18 cartridges, Waters, Milford, MA, USA) was conditioned thrice with 100% methanol pushed through at a rate of 2 to 3 drops per second via a syringe. The stationary phase was acidified three times with 0.1% FA following the same method as conditioning. The sample was loaded into the column and allowed to flow via gravitational force and the flow-through was reloaded three times. Next, the sample loaded column was desalted twice with 0.1% FA. Elution buffer (75% ACN+0.1% FA) was added and, using a syringe, the buffer was pushed through the column and the sample was collected. This C18 desalting protocol was performed thrice with the desalting wash’s solution and the flow-through combined together. The samples were pooled and placed in the SpeedVac to dry and stored at –20 °C.

2.7. Electrostatic repulsion–hydrophilic interaction chromatography (ERLIC)
Eight hundred μg of iTRAQ-labeled peptides were fractionated using PolyWAX LP weak anion-exchange column (4.6×200 mm, 5 μm, 300 Å; PolyLC, Columbia, MD, USA), within the Shimadzu HPLC system (Kyoto, Japan). The HPLC gradient used composed of 100% solvent A (85% ACN, 0.1% ammonium acetate, 1% FA, pH 3.5) for 5 min, 0%–36% solvent B (30% ACN, 0.1% FA, pH 3.0) for 15 min, and 36%–100% solvent B for 25 min, and finally 100% solvent B for 10 min, running for a total of 1 h at a flow rate of 1.0 ml min⁻¹. A total of 29 fractions were collected and was later reduced to 16 fractions by pooling of samples. The 16 sample tubes were kept in SpeedVac to dry completely. The dried peptides in each sample tube were reconstituted in 100 μl 0.1% FA for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

2.8. LC-MS/MS analysis
The samples were analyzed thrice using a Q-Star Elite mass spectrometer (Applied Biosystems/MD5 SCIEX; Applied Biosystems, Foster City, CA, USA) coupled with an online micro-flow HPLC system (Shimadzu). Thirty μl of peptide mixture was injected and separated on a home-packed nanobore C18 column with a picofrit nanospray tip (75 μm i.d. x 15 cm, 5 μm particles) (New Objectives, Wubrun, MA, USA) for each analysis. The samples were separated at a constant flow rate of 30 μl/min with a splitter achieving an effective flow rate of 0.3 μl/min. Data acquisition was performed in the positive ion mode, with a selected mass range of 300–1600 m/z, and peptide ions with +2 to +4 charge states were subject to MS/
MS. The three most abundant peptide ions above 5-count threshold were selected for MS/MS and each selected target ion was dynamically excluded for 30 s with 30 mDa mass tolerance. Automatic collision energy and automatic MS/MS accumulation were used to activate smart information-dependent acquisition (IDA). With maximum accumulation time being 2 s, the fragment intensity multiplier was set to 20. The peak areas of the iTRAQ reporter ions reflected the relative abundance of the proteins in the samples.

2.9. Mass spectrometric data analysis

The data was acquired with the Analyst QS 2.0 software (Applied Biosystems/MDS SCIEX). Using ProteinPilot Software 3.0, Revision Number: 114732 (Applied Biosystems), protein identification and quantification were performed. The peptides were identified by the Paragon algorithm in the ProteinPilot software and the differences between expressions of various isoforms were traced by Pro Group algorithm using isoform-specific quantification. The parameters used for database searching were defined as follows: (i) Sample Type: iTRAQ 4plex (Peptide Labeled); (ii) Cysteine alkylation: MMTS; (iii) Digestion: Trypsin; (iv) Instrument: QSTAR Elite ESI; (v) Special factors: None; (vi) Species: None; (vii) Specify Processing: Quantitate; (viii) ID Focus: biological modifications, amino acid substitutions; (ix) Database: concatenated ‘target’ (IPI rat; version 3.40; 40389 sequences and 20,549,266 residues) and ‘decoy’ (the corresponding reverse sequences for false discovery rate (FDR) estimation); and (x) Search effort: thorough. Pro Group algorithm was used to automatically select the peptide for iTRAQ quantification, where the reporter peak area, error factor (EF) and P-value were calculated. Auto bias-correction was carried out on the acquired data to remove variations imparted as a result of unequal mixing during the combination of the differently labeled samples. To minimize the false positive identification of proteins, a strict cutoff of unused ProtScore ≥ 2 was used as the qualification criteria, which corresponds to a peptide confidence level of 99%. A FDR of 0.33% (<1.0%) was achieved. As the qualification criteria, which corresponds to a peptide confidence level of 99%. A FDR of 0.33% (<1.0%) was achieved. The cutoff for up- or down-regulation (pre-defined at 1.2 and 0.83 respectively) was determined by using the P-value cutoff of 0.05 to obtain the list of proteins with significant ratios. The P-value assigned by the ProteinPilot software measures the confidence of the real change in the protein expression level. Then data analysis and functional classification were conducted using online databases such as NCBI, UniProt, and Panther.

2.10. Post-proteomic data verification SDS-PAGE and western blot analysis

Twenty micrograms of cell lysates were resolved by 8–12% SDS-PAGE at 0.02 Ampere (A) of constant current and transferred to a polyvinylidine fluoride (PVDF) membrane (0.22 µm; Amersham) using the ‘semi-dry’ transfer method (BioRad, Singapore) for 60 min at 0.12 A in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% (wt/vol) SDS. The membrane was blocked with 5% BSA (BioRad) in Phosphate-buffered saline (PBS) plus 0.1% Tween-20 (PBS-T) for 2 h at RT, washed three times in PBS-T for 10 min each, and incubated with primary antibody (diluted in 2% BSA in PBS-T) for overnight at 4 °C. The membranes were washed as described above, incubated with HRP-conjugated secondary antibody for 1 h at RT, and developed using the ECL plus western blot detection reagent (Amersham). X-ray films (Konica Minolta Inc., Tokyo, Japan) were exposed to the membranes before film development in a Kodak X-OMAT 2000 processor (Kodak, Ontario, Canada). For equal sample loading, protein quantification was performed using a ‘2D Quant’ kit (Amersham) with at least two independent replicates. BSA was used as a standard for protein quantification. To re-probe the same membrane with another primary antibody, Pierce’s (Pierce Biotechnology, Inc., Rockford, IL, USA) ‘stripping solution’ was used to strip the membranes. In addition, equal sample loading was confirmed using Gapdh as a reference protein. Western blot experiments were performed at least four times for statistical quantification and analyses (n=4), and representative blots are shown. Values (± relative protein expression) represent the ratio of densitometric scores (GS-800 Calibrated Densitometer and Quantity One quantification analysis software version 4.5.2; BioRad) for the respective western-blot products (mean±SD (standard deviation)) using the Gapdh bands as a reference.

2.11. Statistical evaluation

The mechanical responses of the vessels were measured as force and expressed as active wall tension, which is the increase in measured force divided by twice the segment length [34]. By using a computer program (GraphPad, Institute for Scientific Information, San Diego, CA, USA), the concentration-response curves (CRCs) were fitted to the classical Hill equation, as described earlier [35]. The results are expressed as mean±SD. Differences between means were analyzed using either one-way analysis of variance (ANOVA) followed by a Bonferroni t-test, Student’s t-test or paired t-test (SPSS (Statistical Products and Service Solutions) for Windows Version 19 was used to perform ANOVA, optionally followed by Fisher’s Protected Least Significant Difference (PLSD) post hoc tests, when warranted). For the western blot analyses the Student’s t-test was applied accordingly. For the iTRAQ analysis ProteinPilot Software 3.0 was used as described above. To be considered statistically significant, we required a probability value to be at least <0.05 (95% confidence limit, *P<0.05).

3. Results

3.1. Tianma-induced proteome profile changes in rat brain tissue

In order to comprehensively understand how tianma regulates regenerative processes in the brain, iTRAQ analysis was performed on the purified protein extracts from rat brains with or without tianma treatment. Four samples set into two batches (each batch (BI and BII) contained six tianma-treated samples and six untreated/control samples, thus 12 controls and 12 tianma-treated samples) were subjected to iTRAQ analysis in order to ensure the results were statistically meaningful. The quality of the dataset and instrumental reproducibility was then confirmed by comparing and combining three technical
replicates after the samples were labeled with 114, 115, 116 and 117 isobaric tags and processed in LC–MS/MS. Identified proteins were also visualized in a virtual two-dimensional protein gel, JVirGel, and categorized by their calculated isoelectric points and molecular weights to ensure the full coverage of the brain proteome [36]. The protein spots were well separated without aggregation, indicating a well-qualified whole brain tissue cell proteomic pattern (Fig. 2).

Comparing the expression levels between tianma-treated and untreated samples, we identified a total of 710 proteins through iTRAQ, whereby 495 proteins were quantified (with a strict cutoff of unused ProtScore ≥ 2 as the qualification criteria, which corresponds to a peptide confidence level of 99% and an applied FDR of 0.33% (<1.0%), out of which 58 showed an altered protein expression level (26 proteins were down-regulated and 22 proteins were up-regulated, Supplementary Table 1); among those proteins, five proteins were significantly changed (P < 0.05): Arf3, Pacsin1, Gnao1, Dctn2 and Anxa5 (Table 1). By using online databases (Panther, UniProt, and NCBI) for classification, of the 495 proteins, about 40% were sub-grouped as enzymes with catalytic activities, about 19% were related to structural molecule activities and about 6% were receptor proteins (Fig. 3).

To our interest, a larger set of the modulated proteins was related to NDs such as Arf3, Pacsin1 (both with P < 0.05), Ap2b1 (P < 0.08) and Tubb2c/3/5 (to HD); Sncb, Park7, Ndufb6/8, Nefl and protein 14-3-3e (to PD) (Fig. 3, Supplementary Table 1). In this context it was also of interest to see the changes of proteins such as Gnao1, Dctn2 and Anxa5 (all with P < 0.05) as well as changes for Sirpa (P < 0.08), Hsp90b1 (P < 0.08), Glud1 (P < 0.06) and Gar1 (P < 0.06).

### 3.2. Validation of the changes of protein expressions by tianma treatment by western blot

Following the database search and classification of proteins, western blots were performed on randomly selected proteins to further verify the iTRAQ results. As shown in Fig. 4, Ncam1, Dctn2 and Sncl were down-regulated by tianma treatment, whereas the Anxa5, Pacsin1 and Arf3 protein levels were up-regulated. Thus, the western blot results were consistent with the iTRAQ data obtained.

### 3.3. STRING protein–protein interaction analysis of tianma-affected proteins

STRING (Search Tool for the Retrieval of Interacting Genes) is a database resource dedicated to protein–protein interactions, including both physical and functional interactions. It weighs

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### Table 1 – Functional classification of differentially expressed proteins between control and tianma-stimulated rat brain quantified by iTRAQ-based proteomics.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Proteins name/genes symbol</th>
<th>Molecular function</th>
<th>No. of peptides (&gt;95%)</th>
<th>T:C iTRAQ-ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huntington Disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPI00231674</td>
<td>ADP-ribosylation factor 3; Arf3</td>
<td>GTPase activity</td>
<td>3</td>
<td>6.43</td>
<td>0.04960</td>
</tr>
<tr>
<td>IPI00208245</td>
<td>Protein kinase C and casein kinase substrate in neurons protein 1; Pacsin1</td>
<td>Cytoskeleton organization</td>
<td>10</td>
<td>3.40</td>
<td>0.02001</td>
</tr>
<tr>
<td>Neuronal development</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPI00204843</td>
<td>Guanine nucleotide-binding protein G (o) subunit alpha; Gnao1</td>
<td>GTPase activity</td>
<td>9</td>
<td>0.34</td>
<td>0.00930</td>
</tr>
<tr>
<td>IPI00213015</td>
<td>Dynactin subunit 2; Dctn2</td>
<td>Microtubule motor activity</td>
<td>2</td>
<td>0.40</td>
<td>0.01291</td>
</tr>
<tr>
<td>Molecular chaperon/response to stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPI00471889</td>
<td>Annexin A5; Anxa5</td>
<td>Calcium ion binding</td>
<td>3</td>
<td>6.37</td>
<td>0.01779</td>
</tr>
</tbody>
</table>

The list contains quantitative information of the proteins from tianma-stimulated rat brain compared with control. These proteins have met the criteria (i.e., unused ProtScore > 2.0, FDR = 0.33% (<1.0%), change in expression levels of at least 1.2-fold (up-regulation) or at least < 0.83-fold (down-regulation)) as defined in the experimental procedures.

a The total number of peptides identified with >95% confidence; C = control cells, T = tianma-treated cells.
and integrates information from numerous sources, including experimental repositories, computational prediction methods, and public text collections, thus acting as a meta-database that maps all interaction evidence onto a common set of genomes and proteins [37]. This analysis provides an essential system-level understanding of cellular events in a functional brain. Functional partnerships between proteins are at the core of complex cellular phenotypes, and the networks formed by interacting proteins provided us with crucial scaffolds for modeling and data reduction to get insight into the potential mechanisms of how tianma affects brain functions. In this study, the STRING analysis revealed the functional links among the various significantly regulated proteins (Pacsin1, Gnao1, Arf3, Anxa5 and Dctn2) and their potential connections to other metabolically modulated proteins (such as Ap2b1, Mbp, Sncb, 14-3-3e (YWHAE), Sirpa) as well as ion channels (e.g. Vdac2 and Kcnj 11) that might play a pivotal role in neuro-synaptic and neuro-regenerative processes (Fig. 5).

3.4. IPA signaling pathway analysis of tianma-modulated proteins

Further, we used Ingenuity Pathways Analysis (IPA) to bio-computationally analyze the potential networks of the significantly regulated proteins in tianma-treated brain tissue. This analysis provided us additional valuable clues about the complex interactive activities of the various significantly regulated proteins and other identified proteins for their commonly known interactive protein networks as well as for other cellular metabolic information and potential links to various NDs including HD (Fig. 6).

4. Discussion

HD is an autosomal dominant hereditary neurological disorder caused by an expansion of triplet repeats in the gene for the protein huntingtin. A current view of the molecular and cellular defects associated with this disease is that the abnormal huntingtin protein leads to changes in vesicle trafficking in neurons [38-41]. Among the proteins regulated in tianma-treated brains were a number of proteins related to synaptic plasticity and HD signaling pathways.
4.1. Tianma, neuronal growth cone control, synaptic activities and HD

Dynactin is a multi-subunit complex that serves as a critical cofactor of the cytoplasmic microtubule motor dynein and plays an essential role in microtubule-dependent axonal transport and related growth cone advance. Alpha-E-catenin functions as a dynamic link between the dynactin complex and actin, thus integrating the microtubule and actin cytoskeleton during intracellular trafficking [42–44]. Due to the changes in Dctn2, tianma could interfere with and support the neuronal intracellular trafficking system, which is particularly important when temporal and spatial modulations (increase or decrease) may contribute to synaptic plasticity.

G(o) is the most abundant G-protein in the brain, where it is involved in metabotropic glutamate receptor as well as opioid receptor signaling mediated via extracellular signal-regulated kinase activation [45–47]. Gnao1 is a guanine nucleotide binding protein which is expressed during neurite extension processes [48]. It co-localizes with the G-protein-regulated inducer of neurite outgrowth 1 protein (Grin1), suggesting that the Gnao–Grin1 pathway could mediate significant roles in neuronal migration and differentiation at embryonic stages and exert functions in wiring and/or maintenance of specific neural circuits at postnatal to adult stages [49,50]. By its down-regulation, tianma may centralize specific neurotransmission pathways that are important during synaptic plasticity, and could also use these signaling pathways to inhibit hippocampal epileptiform activity [6,51–53].

ADP-ribosylation factors (ARFs) are highly conserved approximately 20-kDa guanine nucleotide-binding proteins that play crucial roles in neuronal intracellular vesicular transport and in regulations of phospholipid-modifying enzyme activities and cytoskeletons [54,55]. Findings by Suzuki et al. suggest that ARFs are differentially involved in some processes essential to nerve regeneration as well as neuronal differentiation and maturation [56]. Thus, the regulatory influence of tianma on Arf3 could have a wider impact on neuronal differentiation (e.g. neural stem cells activated and recruited to affected areas upon nerve injury) and neuronal regeneration in the central nervous system (CNS).

Pacsins are highly conserved Src-homology 3 (SH3)-domain-containing proteins that seem to exist in all multicellular eukaryotes. They interact with the large GTPase dynamin and several other proteins implicated in vesicle trafficking. Pacsin1, which is mainly detected in brain tissue, is one of
the Pacsin-family proteins involved in endocytosis and recruitment of synaptic vesicles. Pacsins are intracellular adapter proteins involved in vesicle transport, membrane dynamics and actin reorganization [57]. Pacsin1 binds to dynamin, synaptojanin-1 and N-WASP, and functions in vesicle formation and transport [58–61]. It is up-regulated upon neuronal differentiation [62]. Particularly, up-regulated Pacsin1 links the NMDA receptor subunit NR3A to the clathrin-dependent endocytosis machinery and probably serves to clear NR3A-containing NMDA receptors from maturing active glutamatergic synapses [63]. Furthermore, due to its binding to the huntingtin protein and the finding of reduced immunostaining in synaptic varicosities beginning in presymptomatic and early-stage of HD, it has been suggested that Pacsin1 may play a role during early stages of the selective neuropathology of HD. Mutant huntingtin affects proteins involved in synaptic function and cytoskeletal integrity before symptoms develop which may influence early disease onset and/or progression [64,65]. Interestingly, it has been demonstrated very recently that tianma prevents huntingtin aggregations through activation of the adenosine A (2A) receptor and the ubiquitin proteasome system [66]. Accordingly, considering the various points mentioned so far, tianma might be considered for the early stage treatment of HD subjects.

4.2. Tianma, molecular chaperons and the misfolded protein response

Previous studies have implicated annexins in forming scaffolding platforms at cell membranes, thus contributing to a decrease in their dynamics and ion channel regulation, in particular Anxa5 in the traffic of the cystic fibrosis transmembrane conductance regulator (CFTR) [67,68]. Interestingly, recently it was demonstrated that Anxa5 can prevent the aggregation of misfolded proteins (such as amyloid beta peptide or alpha-synuclein) and thus might act as a molecular safeguarding chaperon against the formation of toxic amyloid...
aggregates in AD or PD [69]. Additionally, it could eventually protect against ischemic insults in the brain [70,71]. Thus, the tianma-induced increase in Anxa5 may have neuroprotective functions that could be beneficial for the prevention of aging-related diseases associated with misfolded proteins such as AD, PD or HD.

5. Conclusions

We investigated the in vivo effect of tianma on potential molecular and cellular mechanisms involved in tianma-mediated brain functions and neuroregenerative processes in the CNS. According to our study, the long-term treatment with tianma at low doses could influence the entire brain proteome with potential neuro-restorative effects possibly important for the efficient therapeutic treatment of NDs such as HD. However, since tianma contains many bioactive ingredients, a more systemic biological study is necessary to understand the cerebrovascular functions of herbs such as tianma and to unravel the medically active components in tianma. The disclosure of the pharmacological actions of those components would facilitate the application of this herb as a food supplement or medicine in the clinic [8,22,33,72,73].

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2012.02.029.

Acknowledgment

This study was supported by the Institute of Advanced Studies, Nanyang Technological University.

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