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Neurotrophic Effects of Tianeptine on Hippocampal Neurons: A Proteomic Approach

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Tianeptine, an atypical tricyclic antidepressant with unique characteristics, can improve memory and prevent stress-induced hippocampal damage. It has neuroplastic and neurotrophic effects on hippocampal neurons and can prevent dendritic atrophy of the hippocampus in certain pathological conditions. To obtain a better understanding of the underlying mechanisms, we performed a proteomic analysis on tianeptine-treated hippocampal neurons. Primary hippocampal neurons were prepared from fetal Sprague−Dawley rats, eliminating glia cells by addition of cytosine β-D-arabinofuranoside at day 2 *in vitro* (DIV2). The neurons were treated with tianeptine (10 μg/mL) or vehicle at DIV3, then harvested at DIV4 or DIV9 for immunocytochemical analysis of, respectively, neurite outgrowth or synapse formation. A proteomics analysis was performed on DIV4 neurons and the data were confirmed by Western blot analysis. Using specific markers, we demonstrated that tianeptine can augment neurite growth and promote synaptic contacts in cultured hippocampal neurons. The proteomics analysis identified 11 differentially expressed proteins, with roles in neurite growth, metabolism of neurotrophic substances, synaptogenesis, and synaptic activity homeostasis. The data shed light on the mechanisms underlying the neurotrophic effect of tianeptine observed in both animal studies and the clinic.

Keywords: Tianeptine • proteomics • primary hippocampal neuron cultures • neurite outgrowth • synapse formation • 2-D electrophoresis • neurotrophic effects

Introduction

To adapt to environmental stimuli, the hippocampus, a part of the limbic system, undergoes continuous remodeling of its microscopic structures, the dendrites, axons, and synapses. Inadequate remodeling of hippocampal neuron structure underlies the pathogenesis of various psychiatric and degenerative diseases, whereas correct remodeling of these structures plays an important role in the normal function of the hippocampus, such as the formation and consolidation of memory. The hippocampus is extremely susceptible to stress-related damage, such as recurrent depression, head trauma, anorexia nervosa, Alzheimer's dementia, and Parkinsonism,[1−4] and shows atrophic remodeling and also atrophy of the dendrites of hippocampal neurons.[5] This implies that a drug that can modulate remodeling of the structure of hippocampal neurons might not only prevent or reverse the hippocampal atrophy seen in a diseased state, but also improve memory in healthy subjects.

Tianeptine, a drug that acts on a wide area of the limbic system, including the hippocampus and amygdala,[6] is able to prevent stress-induced hippocampus damage and enhance memory consolidation. Tianeptine can reverse stress-associated atrophic changes in the hippocampus, prevent dendritic atrophy,[7,8] and normalize the hippocampal volume.[9] In addition, it can improve memory in various conditions. Animal studies have shown that tianeptine can improve memory in an autoshaping Pavlovian/instrumental learning task,[10] prevent memory loss in aging animals as shown by a discrimination acquisition test,[11] and promote memory retention in a hippocampal partial lesion animal model in an open field water-maze test.[12]

Although several studies have tried to explain the neuroplastic and neurotrophic effects of tianeptine, the underlying mechanisms remain unclear. Although earlier studies found that tianeptine can enhance serotonin reuptake,[13−15] a recent

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report has provided firm evidence that it has similar discriminative stimulus properties to the selective serotonin reuptake inhibitor fluoxetine.[16] Modulation of serotonin transmission is required for tianeptine to enhance memory consolidation.[10] In addition to serotonin mechanisms, tianeptine can also affect glutamatergic transmission,[17] especially in the hippocampal CA3 region.[18] Modulation of glutamate transmission is required for tianeptine to correct stress-induced abnormalities and to enhance long-term potentiation of synapses for memory formation.[18,19] Moreover, tianeptine has anticonvulsant activity caused by modulation of hippocampal adenosine A1 receptors[20−22] and can exert neurotrophic effects by upregulation of brain-derived neurotrophic factor,[6] a growth factor mainly secreted by glia cells.[23] Recent studies have suggested that it is the neuroplastic and neurotrophic effects of tianeptine that play a key role in the treatment of depression.[24] However, these results were obtained in *in vivo* studies, and it is not clear whether tianeptine can exert its effects by acting directly on hippocampal neurons in the absence of glial cell-derived factors. To address these questions, an *in vitro* study using cultured hippocampal neurons is necessary.

Proteomics can be used to simultaneously study the expression of all proteins (the proteome), rather than individual proteins, in a cell, tissue, or organ. In addition, proteomic approaches have been used to explore the molecular mechanisms underlying the effects of various drugs.[25,26] We therefore performed a gel-based proteomic analysis to evaluate the neurotrophic/neuroplastic effect of tianeptine on cultured hippocampal neurons. Using a culture system that excluded almost all glial cells, we demonstrated that tianeptine can directly promote the neuritogenesis and synaptogenesis of hippocampal neurons and regulate the expression of proteins which mediate these neurotrophic effects.

Materials and Methods

Preparation of Primary Hippocampal Neuron Cultures. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Chi Mei Medical Center, Tainan, Taiwan, and were consistent with the ethical guidelines in the "Guide for the Care and the Use of Laboratory Animals", published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1985).

Pregnant Sprague−Dawley rats weighting 300−350 g were obtained from the National Yung Ming Medical School, Taipei, Taiwan and were maintained in a constant temperature room, with a light/dark cycle of 12/12 h and with *ad libitum* access to food and water. The 10−16 fetuses from a single pregnant rat were used in one set of immunocytochemical, Western blot and proteomics analyses and a total of six pregnant rats were used. At gestational day 18 or 19, the pregnant rats were euthanized and the fetuses delivered by Cesarean section. The fetal hippocampi were then dissected and neuron cultures were prepared as described previously.[27] Briefly, the dissected hippocampi were cleaned of meninges, collected in Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY), and the tissues were dissociated by digestion with 0.25% trypsin (Gibco) in HBSS for 15 min at 37 °C and trituration with a fire-polished Pasteur pipet. The dissociated cells were counted and plated at a density of 100 cells/mm^2 onto 18-mm poly-D-lysine-coated glass coverslips for immunocytochemistry or at a density of 400 cells/mm^2 onto 10-cm poly-D-lysine-coated Petri dishes for proteomics and Western blot analyses. The neurons were maintained in neural basal media supplemented with B-27 (Invitrogen, Carlsbad, CA), 0.5 M glutamine, and 12.5 μM glutamate at 37 °C in 5% CO_2 in air and were treated at day 2 *in vitro* (DIV2) with cytosine β-D-arabinofuranoside (Sigma-Aldrich, St. Louis, MO) at a final concentration of 2 μM to suppress the proliferation of glial cells.

Drug Treatment. The day of plating was counted as DIV0. For immunocytochemical analyses, after 24 h, when the cells had attached to the substrate, the poly-D-lysine-coated glass coverslips were inverted in the wells of a 24 well culture dish. The hippocampal neurons were then treated with tianeptine (10 μg/mL) (Sigma-Aldrich, St. Louis, MO) or vehicle (0.9% saline) at DIV3. This concentration (10 μg/mL) has been shown to have significant neurotrophic effects on cultured hippocampal neurons (our preliminary study) and to have neuroprotective effects.[28,29] The neurons on the coverslips were fixed at DIV4 in cold methanol for morphological analyses of axonal and dendritic growth or at DIV9 for analysis of synapse formation. For proteomics or Western blot analysis, neurons cultured in Petri dishes were harvested at DIV4 and lysed with either 2-DE lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 0.5% IPG buffer, pH 3−10) for proteomics or RIPA buffer (100 mM Tris, pH 8.0, 300 mM NaCl, 2% Nonidet P-40, 1% sodium deoxycholate, 0.2% SDS) for Western blots.

Immunocytochemistry. To assess the neurotrophic effect of tianeptine on cultured hippocampal neurons, we examined the outgrowth of axons and dendrites at DIV4 and the synaptic density at DIV9 by immunostaining for specific markers as described previously.[30,31] Briefly, neurons on glass coverslips were fixed in methanol at 4 °C, permeabilized in 0.05% Triton X-100 in hypertonic phosphate-buffered saline (HPBS, 20 mM $NaPO_4$, 450 mM NaCl, pH 7.4), rinsed with Dulbecco's PBS (D-PBS, Gibco), and blocked with 5% skimmed milk in HPBS. After blocking, the DIV4 cells were immunostained overnight at 4 °C with mouse antibodies against rat neurofilament-L (NF-L, an axon marker) or rat microtubule-associated protein 2 (MAP-2, a dendritic marker) (both 1:300 in blocking buffer), while the DIV9 cells were double-stained under the same conditions with rabbit antibodies against rat synaptophysin (a presynaptic marker) and a mouse monoclonal antibody against rat MAP-2 (a postsynaptic dendritic marker) (both 1:100 in blocking buffer). All first antibodies were from Sigma-Aldrich. For single immunostaining, the cells were then stained for 1 h at room temperature with FITC-conjugated anti-mouse IgG antibodies diluted 1:50 in blocking buffer, while for double immunostaining, the cells were stained under the same conditions with FITC-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG antibodies (Jackson Immuno-Research Laboratories), both diluted 1:50 in blocking buffer. The fluorescence images were captured using a fluorescence microscope (BX-51,Olympus, Tokyo, Japan) equipped with a Olympus digital camera (DP70, Olympus, Tokyo, Japan) using 2040 × 1536 pixels.

Quantification of Neurite Outgrowth and Synaptic Contacts (Synaptic Formation). For the morphological analysis of dendritic and axonal outgrowth, 200 anti-MAP-2 antibody-stained neurons and 200 anti-NF-L antibody-stained neurons were used (100 each from the control and tianeptine-treated groups); the neurons were randomly selected from the stored data from the 6 independent experiments. The axonal length per neuron and dendritic length per neuron were measured and the number of dendritic branch tips per neuron counted as described previously.[30,31] Briefly, a dendrite was defined as a tapered anti-MAP-2 antibody-stained process more than 20

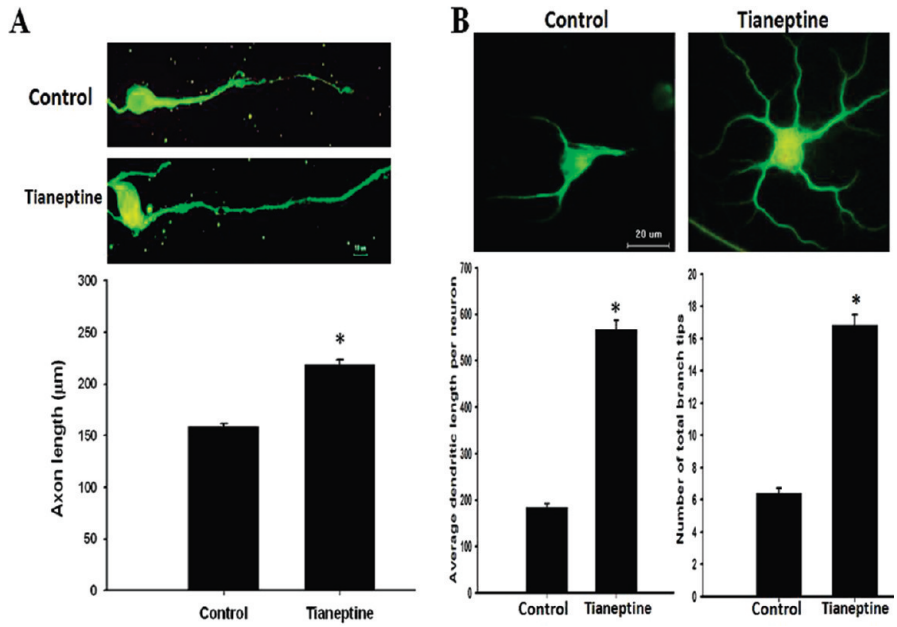

Figure 1. Estimation of the growth of axons and dendrites in control and tianeptine-treated hippocampal neurons. Cultured primary hippocampal neurons were treated with vehicle (control) or tianeptine (10 μg/mL) on DIV3, then stained on DIV4 with anti-NF-L and anti-MAP-2 antibodies to identify, respectively, axons and dendrites. (A) Representative photographs of stained axons from both groups are shown in the upper panel and the statistical data for axon length are shown in the lower panel. (B) Typical photographs of stained dendrites are shown in the upper panel and bar charts for average dendrite length (left) and branch tip number (right) are shown in the lower panel. Scale bar, 10 μm. The data are the mean ± SEM (100 neurons per group). *$P < 0.05$, compared using Student's t test.

μm in length, and the total length of the dendritic arbor per cell was determined by calculating the sum of the lengths of individual dendrites on the cell. The dendritic branching tips were counted to represent the complexity of arborization. An axon was defined as the longest anti-NF-L antibody-stained neurite. The above data were obtained using PC-based image analysis software (Image Pro 3.0 Plus, Media Cybernetics Silver Spring, MD).

To evaluate synapse formation in the control and tianeptine-treated groups, additional 200 neurons (100 control and 100 tianeptine-treated) were randomly selected from DIV9 neurons double-stained with anti-synaptophysin and anti-MAP-2 antibodies. The number of axon-dendritic contacts along an innervated anti-MAP-2 antibody-stained dendrite was estimated by manually counting the presynaptic specializations on neuron images as described previously.[30,31] A punctum was taken as a roughly spherical, brightly synaptophysin-stained spot (red) juxtaposed with an anti-MAP antibody-2-stained (green) dendrite. Synaptic density was determined by calculating the number of synaptophysin puncta per 50 μm length of dendrite using Image Pro 3.0 Plus software (Media Cybernetics Silver Spring, MD).

Western Blot Analysis. Western blot analysis was performed on DIV4 neuron cultures ($n = 6$ for each group) to confirm the changes in expression of some proteomics-identified proteins using a previously described protocol .[32] Briefly, following two rinses with ice-cold PBS, the cells were scraped off the culture dishes and lysed in 100 μL of ice-cold RIPA buffer. Cell debris was removed by centrifugation at 15,000g for 15 min at 4 °C and the protein concentrations of the lysates measured using a Bio-Rad protein kit (Bio-Rad Lab, Hercules, CA). An equal volume of 2× reducing SDS sample buffer was added to the lysates and the mixture heated at 95 °C for 5 min. The samples (30 μg of protein/gel lane) were subjected to 10% SDS-PAGE and the proteins transferred to a Hybond PVDF membrane (GE Healthcare, NJ). The membrane was blocked at room temperature for 1 h in 20 mL of blocking buffer [Tris-buffered saline, pH 8.0, containing 0.05% Tween-20 (TBST) and 5% skimmed milk], then incubated overnight at 4 °C with primary antibody diluted in blocking buffer, washed with TBST four times, and incubated for 1 h at room temperature with appropriate secondary antibody diluted in blocking buffer. The primary antibodies used were rabbit antibodies against rat syntenin-1 (1:5000, Millipore), rat CRMP-2 (1:5000, Chemicon), rat SNAP-29 (1:500, Santa Cruz), or rat calpain 1 (1:500, Epitomics) or mouse antibodies against rat GAPDH (1:5000, Chemicon), while the secondary antibodies used were goat anti-rabbit or rabbit anti-mouse IgG antibodies (1:10,000, Chemicon). Bound antibodies on the membrane were visualized with an ECL detection kit (Millipore, Bedford, MA), and the intensity of the stained band was quantified using a densitometer and normalized to the intensity of the band for the internal control GAPDH.

Preparation of Protein Samples and 2-DE. Harvested neuronal cells at DIV4 were sonicated in 2-DE lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 0.5% IPG buffer pH 3−10) and the lysates centrifuged at 15,000g for 15 min to remove cell debris. The protein concentration in the supernatants was determined by a modified Bradford method.[33]

Proteins (250 μg) from control ($n = 6$) and tianeptine-treated ($n = 6$) neuron cultures were loaded onto IPG strips (Immobiline DryStrip, pH 3−10, GE Healthcare, NJ) for simultaneous rehydration, then isoelectric focusing was performed using the voltage−time program of 50 V for 12 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for a total of 140,000 Vh. Prior to SDS-PAGE, the IPG strips were equilibrated for 15 min in 50 mM Tris, pH 8.4, containing 6 M urea, 2% SDS, 1% DTT, and 30% glycerol, then for 15 min in 50 mM Tris, pH 8.4, 6 M urea, 2% SDS, 2.5% iodoacetamide, and 30% glycerol. The second-

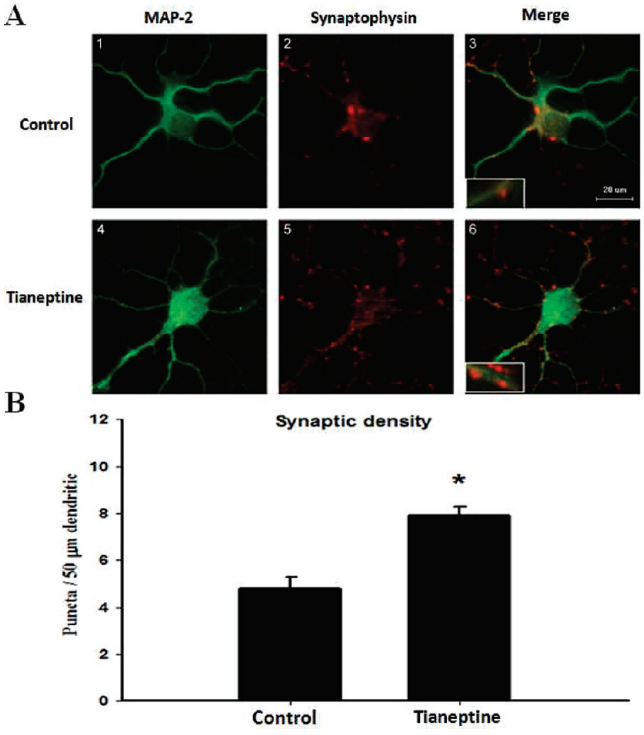

Figure 2. Estimation of synapse formation in control and tianeptine-treated hippocampal neurons on DIV9. (A) Representative photographs of staining of neurons from both groups for the postsynaptic dendritic marker MAP-2 (green) (left panels), the presynaptic marker synaptophysin (red) (center panels), and the merged images (right panels). Scale bar, 20 μm. (B) Quantification of synaptic contacts (synaptophysin-positive puncta) on the dendrites. The data are the mean ± SEM (100 neurons per group). *P < 0.05, compared using Student's t test.

dimension separation was run for 6 h at 15 °C using a vertical electrophoresis system (GE Healthcare, NJ) on 12.5% gels at 20 mA/gel, then the gels were fixed and stained using Sypro-Ruby fluorescent dye according to the manufacturer's instruction (Invitrogen, Carlsbad, CA) and scanned using a Typhoon Trio laser scanner (GE Healthcare, NJ).

In-Gel Digestion and MADLI-TOF MS. Protein spots showing significant differences in expression between vehicle- and tianeptine-treated neurons were manually excised and subjected to in-gel digestion. The digested samples were extracted and analyzed using a MALDI-TOF MS (Autoflex II, Bruker Daltonics, Bremen, Germany). For peptide mass fingerprinting (PMF), the mass spectrum was obtained from signals generated from at least 500 laser shots. The mass spectra were processed using Flexanalysis software (Bruker Daltonics, Bremen, Germany) and mass (monoisotopic mass) lists were obtained using Biotools software (Bruker Daltonics, Bremen, Germany) and used to search the UniProt database (http://www.pir.uniprot.org) using the MS-Fit database searching engine (http://prospector.ucsf.edu/mshome.htm). For each PMF search, the mass tolerance was set at 100 ppm and one missed tryptic cleavage was allowed.

Statistics. For the 2-DE data, ImageMaster 2D Platinum analysis software (GE Healthcare, NJ) was used for spot detection, quantification, and comparisons. Student's t test was used for the statistical analysis of neurite outgrowth and synaptic contacts (100 neurons per group), while the two-tailed Wilcoxon rank-sum test was used to compare the spot relative volume (% Vol) of proteins in 2-DE gels and the normalized band intensities (test protein/GAPDH ratio) in Western blots of proteins from control and tianeptine-treated neuron cultures (6 cultures per group). Differences were considered significant at $p < 0.05$. The data are presented as the mean ± SEM.

Results

Tianeptine Increases Neurite Outgrowth at DIV4. Axon and dendrite outgrowth was seen at DIV4. Using NF-L as an axon-specific marker, axon outgrowth was estimated by measuring the average axon length of the neurons. As shown in Figure 1A, tianeptine-treated hippocampal neurons showed a significant increase in axon length compared to control neurons, showing that tianeptine promotes the growth of axons. Similarly, using MAP-2 as a dendrite-specific marker, the shape of the dendrites was observed and dendritic outgrowth estimated. As shown in Figure 1B, tianeptine-treated hippocampal neurons showed a significant increase in dendritic arborization in terms of dendrite length and total branching tips compared to control neurons, showing that tianeptine promotes the growth

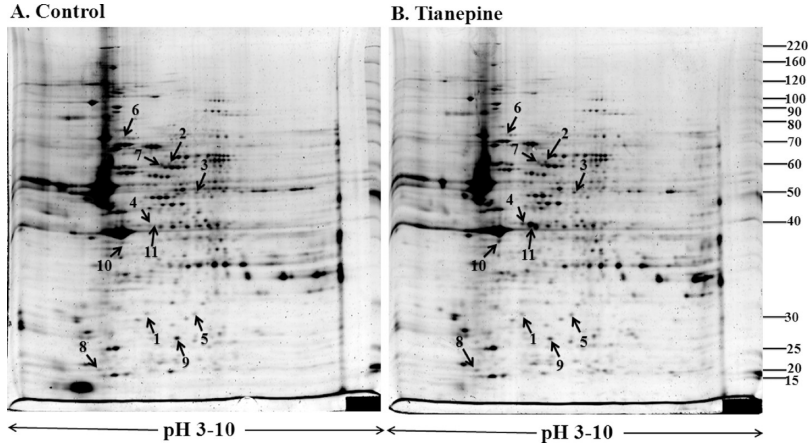

Figure 3. Representative 2-DE gels for control and tianeptine-treated hippocampal neurons. (A) Control cells; (B) tianeptine-treated cells. Differentially expressed proteins are indicated by the arrows and labeled with numbers, which are the same as those in Table 1. The pI range is shown at the bottom of the gels, while the molecular mass is indicated on the right.

Table 1. Identified Proteins[a]

spot no.	MOWSE score	accession no.	protein name abbreviation	protein name	theoretical *Mr* (kDa)/p*I*	observed M_r (kDa)/p*I*	matching peptides	sequence coverage (%)	fold change (+: increase; −: decrease)
1	376	Q9JI92	Syn-1	Syntenin-1	32.4/7.0	30/5.9	6	24.3	+1.3
2	3860000	P47942	CRMP-2	Collapsin response mediator protein 2	62.2/6.0	62/6.5	20	51.9	+2.0
3	11211	Q5XIM9	TCP-1b	T-complex protein 1 subunit beta	57.3/6.0	51/6.7	16	42.8	+1.7
4	21378	P07335	CK-b	Creatine kinase B-type	42.7/5.4	41/5.6	11	39.4	+2.6
5	256	P18297	SR	Sepiapterin reductase	28.1/5.6	29/6.7	5	27.9	+1.8
6	753	Q9JIX5	BCBP	Beta-catenin binding protein	80.6/6.5	76/5.6	10	13.9	−2.5
7	130	Q8CGZ2	ADIP	Afadin- and alpha-actinin-binding protein	70.7/6.2	64/6.2	20	34.1	−2.2
8	2220	Q9Z2P6	SNAP-29	Synaptosomal- associated protein 29	29/5.3	22/5.1	5	33.5	+1.6
9	6818	Q64537	Capns-1	Calpain small subunit 1	28.5/5.3	27/6.5	5	24.4	−1.6
10	15370	P59215	Gnao2	Guanine nucleotide-binding protein G(o) subunit alpha 2	39.9/5.3	38/5.4	13	50.6	+1.8
11	1824	Q5M929	Rnf135	Ring finger protein 135	46/5.5	41/6.1	7	30.1	+14.3

[a] The spot number, MOWSE score, UniProt database accession number, protein name abbreviation, and protein name are shown, followed by the theoretical and observed molecular mass (M_r) and isoelectric point (p*I*). The number of matching peptides and sequence coverage were calculated using Biotools software. The fold change in protein expression is also indicated.

of dendrites. These results show that tianeptine-treated neurons develop a more robust axodendritic network than control neurons.

Tianeptine Increases Pre- and Postsynaptic Contacts at DIV9. DIV9 neurons showed considerable synaptogenesis. Synaptic density was evaluated by double-staining using anti-MAP-2 and anti-synaptophysin antibodies (Figure 2) and merging the images (merge in Figure 2). Puncta were observed as roughly spherical, brightly synaptophysin-stained spots (red spots) juxtaposed with an anti-MAP-2 antibody-stained dendrite (green processes). As shown in Figure 2, there was a significant increase in the number of MAP-2-positive puncta interacting with synaptophysin-positive puncta in tianeptine-treated hippocampal neurons compared to control neurons. Quantitative analysis showed that tianeptine significantly increased the number of contacts made by presynaptic terminals on each dendrite.

Protein Expression Profiles of Control and Tianeptine-Treated Hippocampal Neurons. A 2-DE-based proteomic analysis of control and tianeptine-treated hippocampal neurons was performed at DIV4 to explore changes in protein expression associated with tianeptine-induced neurite outgrowth and synaptogenesis. Overall, the 2-DE gel protein spot patterns of control cells and tianeptine-treated cells were similar, but there was a significant difference in the expression of some individual proteins (Figure 3).

A total of 11 proteins showed significant up- or down-regulation of expression (indicated by the arrows in Figure 3) due to tianeptine treatment were unequivocally identified by the PMF. The characteristics of the identified proteins are listed in Table 1. Figure 4 shows magnified spot images of the identified proteins (Figure 4A) and the relative intensity (percentage spot volume) of each protein spot (Figure 4B). The proteins were syntenin-1 (Syn-1), collapsin response mediator protein 2 (CRMP-2), T-complex protein 1 (TCP-1), creatine kinase (CK-b), sepiapterin reductase (SR), beta-catenin-binding protein (BCBP), afadin- and alpha-actinin-binding protein (ADIP), synaptosomal-associated protein 29 (SNAP-29), calpain (Capns-1), guanine nucleotide-binding protein G(o) subunit alpha 2 (Gnao2), and Ring finger protein 135 (Rnf135). These proteins are known to play roles in neurite growth, cytoskeleton reorganization, metabolism of neurotrophic biomolecules, synaptogenesis, and synaptic functions.

Confirmation of Proteomics Findings by Western Blot Analysis. To confirm the proteomics data, Western blot analysis was used to check the expression on DIV4 of 4 proteins (Syn-1, CRMP-2, SNAP-29, and Capns-1) randomly selected from Table 1. The results obtained showed that the trend for differences for these proteins on Western blots between the control and tianeptine-treated neurons was consistent with the proteomics data (compare Figures 5 and 4).

Discussion

Although tianeptine clearly has neuroplastic and neurotrophic activity, two questions remained unanswered about its mode of action. First, it was unclear whether tianeptine could exert neurotrophic effects on hippocampal neurons when most glia cells, the source of various growth factors, were eliminated. Second, it was not known whether tianeptine regulated the expression of various neuronal proteins and, if so, whether these were responsible for its neurotrophic effects. In the present study, we clearly demonstrated that tianeptine enhanced both neurite growth and synapse formation in cultured hippocampal neurons after most of the glia cells had been eliminated (Figures 1 and 2). Our proteomic analysis also identified 11 proteins (Figures 3−5) which might play roles in the neurotrophic effects of tianeptine (Figure 6).

A well-known neurotrophic effect of tianeptine is to reverse the dendritic atrophy of hippocampal neurons caused by stress or other damage.[7,9,24] This phenomenon is supported by our *in vitro* data, showing that tianeptine treatment promoted neurite growth in cultured hippocampal neurons (Figure 1). Among the identified proteins, Syn-1 (spot 1) and CRMP-2 (spot 2) are involved in the outgrowth of, respectively, dendrites and axons. Syn-1, a PDZ domain-containing protein that can

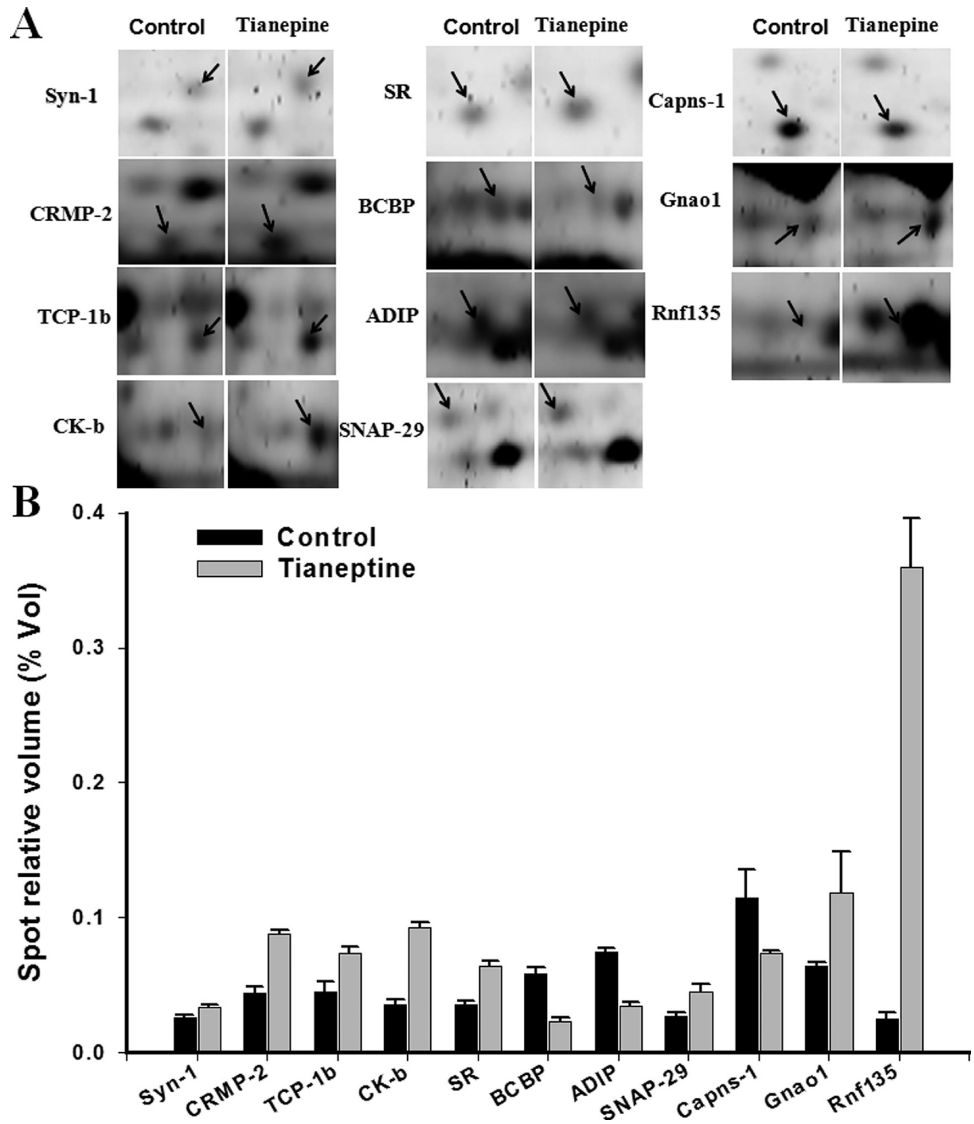

Figure 4. Quantification of differentially expressed proteins. (A) Magnified images of protein spots that are up- or downregulated in hippocampal neurons by tianeptine treatment. (B) Statistical data for the quantification of individual proteins. All differences between the control and tianeptine-treated neurons were significant at the $P < 0.05$ level. The groups were compared using the two-tailed Wilcoxon rank-sum test ($n = 6$).

modulate neuronal function through interactions with various proteins,[34] was upregulated in tianeptine-treated hippocampal neurons compared to control neurons. Since ectopic expression of Syn-1 can increase the number of dendritic protrusions in young and mature neurons,[35] it may be involved in the tianeptine-induced increase in dendrite outgrowth. Similarly, CRMP-2, an axon-specific protein involved in cytoskeleton reorganization[36] for regulating axon growth in both developing and adult brains, also showed significant upregulation in the tianeptine-treated neurons. CRMP-2 may play a role in the tianeptine-induced increase in axon outgrowth, as its overexpression can accelerate axon growth in nerve regeneration.[37] For proper growth of the axon and dendrite, synthesis and reorganization of the cytoskeleton are required. TCP-1, a major neuronal chaperonin,[38,39] is needed for the correct folding of cytoskeletal proteins to support neurite outgrowth.[40] Expression of TCP-1 (spot 3) was upregulated in tianeptine-treated neurons and it might, therefore, play an important role in supporting the tianeptine-induced neurite outgrowth.

Neurotrophic effects are also caused by stimulation by small biomolecules required for neuron survival and neurite growth,[41,42] and the lack of these substances, such as phosphocreatine, is related to stress-induced brain problems.[9] Phosphocreatine is a small biomolecule involved in cellular energy buffering and energy transport,[42] and phosphocreatine supplementation is able to increase cell soma size and the number of neurites in neuronal precursor cells and protect mature neurons from oxidative damage and apoptosis.[41,42] Tetrahydrobiopterin (BH_4), another small molecule, can prevent nitric oxide (NO) toxicity in NO-producing neurons and mediates the nerve growth factor-induced proliferation of PC12 cells.[43,44] CK-b (spot 4) and SR (spot 5) are, respectively, the key enzymes in the biosynthesis of phosphocreatine and BH4,[42,45] and were unequivocally identified in our proteomic analysis. Since tianeptine upregulated the expression of both of these enzymes in neurons, endogenous neurotrophic biomolecules might play a role in the neurotrophic effects of tianeptine. Our *in vitro* data are consistent with previously reported *in vivo* data showing that the stress-induced

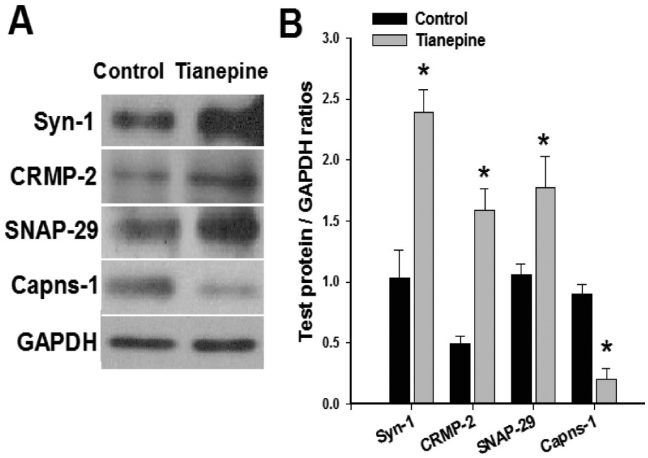

Figure 5. Western blot validation of the expression of some proteomics-identified proteins. (A) Representative blots of Syn-1, CRMP-2, SNAP-29, Capns-1, and GAPDH, showing differences in expression between the two groups (control vs tianeptine). The names of the proteins are indicated on the left. (B) Quantitative data for the indicated proteins. The level of expression of the indicated proteins was normalized to that of the internal control GAPDH. The groups were compared using the two-tailed Wilcoxon rank-sum test ($n = 6$). $*P < 0.05$.

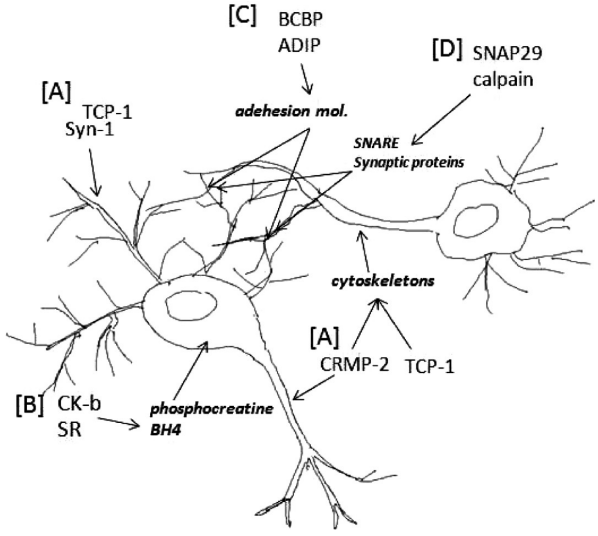

Figure 6. Schematic diagram of the potential effects of tianeptine on cultured hippocampal neurons. (A) Effect of tianeptine on the outgrowth of axons and dendrites, (B) effect of tianeptine on levels of endogenous neurotrophic substances, (C) effect of tianeptine on promoting synapse formation, and (D) functions of tianeptine in maintaining synaptic homeostasis.

reduction in cerebral metabolites (including phosphocreatine) can be reversed by tianeptine treatment.[9]

Synapses are formed by contact and adherence between axons and dendrites, a process that requires the interaction of adhesion molecule complexes located at the pre- and postsynaptic membranes. The cadherin-catenin and nectin-afadin complexes are two adhesion molecule complexes required for synaptogenesis,[46] and their modulation can significantly influence synapse formation.[46,47] BCBP is an adaptor protein that binds to the cadherin-catenin cell adhesion complex to negatively regulate the cadherin-catenin-dependent activation of the transcriptional factor Tcf,[48] while ADIP binds to the nectin-afadin adhesion complex involved in signal transduction.[49,50] In the present study, BCBP (spot 6) and ADIP (spot 7) were downregulated in the tianeptine-treated neurons. Following tianeptine treatment, the downregulation of these adaptor proteins for adhesion complexes might work together to promote synaptogenesis.

Synaptic function is maintained by a delicate control of the activities of synaptic proteins. Uncontrolled neurotransmission (especially glutamate transmission) and calcium influx (especially through NMDA receptors) lead to damage of hippocampal neurons, a common mechanism observed in brain damage caused by neurotoxins or stress.[51] Our data showed that tianeptine can regulate the expression of the synaptic proteins SNAP-29 (spot 8) and calpain (spot 9), possibly to prevent overactivation of synapses. SNAP-29, a SNARE protein that acts as a negative modulator of neurotransmitter release by slowing down the recycling of SNAREs responsible for the exocytosis of synaptic vesicles,[52,53] was upregulated by tianeptine treatment. Thus, the upregulation of SNAP-29 might play a role in preventing overactivation of glutamate transmission. In contrast, calpain, a unique calcium-activated proteinase, was downregulated after tianeptine treatment. Once activated by calcium overloading, it can cause the degradation of many synaptic proteins and contribute to the pathophysiology of neurological disorders.[54,55] The downregulation of calpain might be one way of protecting hippocampal neurons from calcium damage.

Thus, as summarized in Figure 6, tianeptine might have several effects on cultured hippocampal neurons, including effects on the outgrowth of axons and dendrites, levels of endogenous neurotrophic substances, synapse formation, and synaptic homeostasis. Since the present proteomics study focused only on the effects of tianeptine on cultured hippocampal neurons derived from the whole hippocampus of naïve fetal mice, it remains unclear whether tianeptine can reverse proteome changes in adult brain after stress-induced hippocampal damage and whether different subregions of the hippocampus show different tianeptine-induced proteome changes. Future proteomics studies should be performed in stress animal models or in cultured neurons from different subregions to address these issues, as there is evidence that tianeptine can reverse the chronic restraint stress-altered expression of GLT-1 in the CA3 subregion of the rat hippocampus.[56]

Conclusions

In conclusion, we have demonstrated that tianeptine can still exert a neurotrophic effect on hippocampal neurons when most glia cells have been removed. Our proteomic analysis provides an overview of the tianeptine-induced changes in expression of 11 proteins involved in the growth of dendrites and axons, metabolism of endogenous neurotrophic substances, formation of synapses, and homeostasis of synaptic activities. These proteomics data shed light on the mechanisms underlying the neurotrophic effect of tianeptine observed in animal studies and the clinic. Further functional studies are needed to investigate the mechanisms by which these proteins may be involved in the neurotrophic effects.

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