HIPPOCAMPAL SYNAPTIC DYSREGULATION OF EXO/ENDOCYTOSIS-ASSOCIATED PROTEINS INDUCED IN A CHRONIC MILD-STRESSED RAT MODEL

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Abstract—Although major depressive disorder (MDD) is a serious neuropsychiatric illness, its pathogenesis remains unclear. Current evidence suggests that the abnormal transmission and plasticity of hippocampal synapses play an important role in the pathogenesis of MDD. In this study, a two-dimensional gel-based proteomic approach to profile alterations of synaptosome protein expression was applied in the hippocampus of rats subjected to chronic mild stress. Through mass spectrometry and database searching, 19 differentially expressed proteins were identified, of which 5 were up-regulated and 14 were down-regulated in the chronic mild-stressed group as compared with the control group. Subsequently, several proteins of interest were further validated by Western blotting. A detailed analysis of protein functions and disease relevance revealed that synaptic exo/endocytosis-associated proteins may participate in a central mechanism that underlies the abnormal transmission and plasticity of hippocampal synapses found in the chronic mild-stressed rats, and provides guidance to advance our understanding of the pathogenesis of MDD. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: major depressive disorder, chronic mild stress, hippocampus, proteomics, synaptosome, two-dimensional gel electrophoresis.

INTRODUCTION

Major depressive disorder (MDD) is one of the most widespread and serious mental illnesses, affecting more than 120 million people annually worldwide and contributing to increased rates of disability and suicide (WHO, 2001). Despite its severity, the pathogenesis of this affective disorder is poorly understood and remains a challenging scientific problem.

Mounting evidence suggests that the hippocampus, a cerebral cortical structure associated with behavioral inhibition, memory, and spatial coding, plays an important role in MDD pathogenesis. The hippocampus is anatomically connected to several structures in the limbic system which regulates emotional behavior, namely the septum, hypothalamic mammillary body, and thalamic anterior nuclear complex (Campbell and Macqueen, 2004). Ineffective synaptic transmission and disordered synaptic plasticity are widely considered to be the two key underlying factors in the hippocampal dysfunction found in MDD. Clinical studies on MDD patients have reported lower GABA, 5-HT and noradrenaline (NA) levels in the plasma, urine and cerebrospinal fluid (CSF), which supports the deficiency in synaptic transmission found in the brain (Brambilla et al., 2003; Beilmaker and Agam, 2008). These abnormal synaptic transmissions underly the behavioral and cognitive dysfunction in MDD (Sarter et al., 2007). On the other hand, a fundamental property of the synapse is its ability to self-modify the efficacy of its own transmission, which is referred to as synaptic plasticity. Concretely, synaptic transmission was enhanced by synaptic long-term potentiation (LTP) and weakened by synaptic long-term depression (LTD) (Linden, 1999). Recent preclinical and clinical investigations have indicated that stress and aversive experiences can decrease the amount of LTP and enhance LTD in the hippocampus (Popoli et al., 2002;
Holderbach et al., 2007). Moreover, alterations of synaptic plasticity may impair function of brain circuits involved in the pathophysiology of MDD (Holderbach et al., 2007).

From the foregoing studies, it can be concluded that the hippocampal synapses play an important role in the molecular mechanism underlying MDD. Therefore, profiling the protein expression patterns of the hippocampal synapses should help to decipher the pathways and chemical species involved in MDD pathogenesis. In previous proteomic studies on depressed animal models, analysis has mainly focused on protein expression in the whole hippocampus. Thus, due to the complexity of the samples, little information specific to synaptic proteins was discovered (Carboni et al., 2006; Mu et al., 2007; Marais et al., 2009; Kedracka-Krok et al., 2010).

Fortunately, synaptoproteomic technology provides a powerful tool to reduce this proteome complexity. More significant information can be revealed by profiling the differential expression of synaptic proteins under specific pathophysiological conditions. This technology has been widely employed in mental disorder research (Moron et al., 2007; Zhou et al., 2010). However, to date, few studies focusing on the synaptic proteins in animal models of depression have been conducted (Mallei et al., 2008, 2011).

In the present work, differential analysis of hippocampal synaptic proteins using the synaptoproteomic approach was applied in rats subjected to chronic mild stress (CMS), a well-validated model of MDD. After hippocampal synapses were purified down to so-called “synaptosomes” by differential and density-gradient centrifugation, the constituent proteins were extracted and separated by two-dimensional gel electrophoresis (2DE). Simultaneously, the differential spots obtained by PDQuest software were subjected to in-gel digestion and identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight/Time-of-Flight (MALDI-TOF/TOF) mass spectrometry. This study should provide a valuable resource for deciphering the molecular mechanism underlying the abnormal synaptic transmission and plasticity in MDD.

EXPERIMENTAL PROCEDURES

Comparison of three classic grindings in hippocampal synaptosome preparation

After 30 healthy adult male Sprague–Dawley rats (Chongqing Medical University, Chongqing, China) were sacrificed by decapitation and the hippocampi were removed. For differential analysis of the hippocampal synaptosomal proteins, the fresh tissue was homogenized using the motor-operated disperser, and the synaptosome fractions were isolated according to the aforementioned procedure. The following proteome analysis was carried out according to the procedure reported by Grippo et al. (2002) with slight modifications. Briefly, it was performed twice weekly in a 1-h test after food and water deprivation for a 20-h period, which was believed to be the baseline of SP test. The sucrose and water intakes (g) were recorded, and the SP was calculated according to the following formula: SP = sucrose intake (g)/water intake (g). After 3 weeks, the average sucrose intake of each rat was measured. Based on the method of randomized block design, the rats were then divided randomly into CMS and control groups (n = 29 per group) according to the sucrose intakes of each rat in the final SP test. Subsequently, the CMS experiment was performed according to the protocol described by Lewitus et al. with minor modifications (Lewitus et al., 2009). In brief, rats were subjected to a 45° cage tilt, strobeoscopic lighting, intermittent white noise (80 dB), soiling of cages, food and water deprivation followed by restricted food (0.5 g of food pellets), paired housing and overnight lighting. This series of stressors was applied for a 1-week period and repeated for 4 weeks (see Table 1). In the meantime, the SP test was carried out once weekly during the CMS period.

Following these experiments, the rats were sacrificed by decapitation, and the hippocampi were removed. For differential analysis of the hippocampal synaptosomal proteins, the fresh tissue was homogenized using the motor-operated disperser, and the synaptosome fractions were isolated according to the aforementioned procedure. The following proteome analysis was conducted on three sample pools corresponding to the two groups, and each pool originated from nine or ten rats. The experiments were performed in accordance with all regional guidelines and regulations.

Protein sample preparation

The synaptosome fractions were suspended in a lysis buffer containing 7 M urea (Bio-Rad, Hercules, CA, USA), 2 M thiourea (Bio-Rad), 4% 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonate (CHAPS) (Bio-Rad), and 50 mM DTT (Bio-Rad). After 1 h of incubation on ice, the sample was centrifuged at 40,000g for 30 min at 4°C to remove insoluble materials. The protein concentration was estimated using a Bradford protein assay.
assay kit (Bio-Rad, Hercules, CA, USA) with BSA as a standard. Before electrophoresis, the protein sample was processed with a ReadyPrep 2D cleanup kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. The resultant pellet was redissolved in 7 M urea, 2 M thiourea and 4% CHAPS, and the protein concentration was re-determined.

### 2-D electrophoresis and gel image analysis

Samples containing 120 μg of total protein were mixed with a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.2% Bio-Lyte, and 0.001% bromophenol blue (Bio-Rad) to a total volume of 350 μl, and used for the rehydration of 17-cm IPG strips (pH 3–10 NL, Bio-Rad). A multi-step IEF voltage program was applied to the strips on a Protean IEF cell (Bio-Rad): 50 V for 12 h, 250 V for 30 min, 1000 V for 1 h, from 1000 V to 10,000 V over a 5-h step-up period, and 10,000 V for 6 h. Strips were equilibrated in the reduction buffer (0.375 M Tris–HCl pH 8.8, 6 M urea, 20% glycerol, 2% sodium dodecyl sulfate (SDS) and 2% DTT), then in the same buffer but containing 2.5% IAA instead of 2% DTT. The second dimension was accomplished by running the strips on 1 mm-thick 10% SDS–polyacrylamide vertical slab gels using a Protean II xi Multi-Cell (Bio-Rad). The protein condensation and separation were performed at 12.5 mA/gel for 30 min and 25 mA/gel for 5.0–5.5 h at 20 °C. Protein spots were visualized by silver staining according to Yan (Yan et al., 2000). In our experiment, the three samples run in duplicate for technical replicates.

After visualization, images were obtained using an Epson 10000XL scanner (Epson Co., Ltd. Beijing, China) at an optical resolution of 600 dpi. Image analysis and spot detection were accomplished with PDQuest software version 8.0.1 (Bio-Rad Laboratories, Hercules, CA, USA) using Gaussian spot modeling. For quantitative comparisons of spots across gels, six analytical gels (one gel per sample and three gels per group) were analyzed. To correct for the variability in silver staining, the individual spot volumes were normalized by dividing each spot’s optical density (OD) value by the sum total OD of all the spots in the respective gel. Automated and manual spot matching were also performed. Spots showing at least a 1.5-fold change in their integrated intensities in the replicate gels and with a Student’s t-test p-value of < 0.05 were considered to be statistical differences in protein expression between the two groups (Kedracka-Krok et al., 2010).

### Protein identification by MALDI-TOF/TOF

In-gel protein digestion was performed according to Zhou et al. with minor modifications (Zhou et al., 2010). The protein spots of interest were excised from the gels and then destained. After reduction and alkylation, the gel slices were digested overnight with Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA). The digested peptides were extracted with 100 μl 60% CAN (Merck, Darmstadt, Germany) containing 0.1% TFA (Merck) and concentrated in a Speed Vac. The peptides were redissolved using a matrix solution and spotted on a MALDI target plate. Peptides were analyzed using the 4800 Plus MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, USA) in the default mode. The data search was conducted on GPS Explorer (Version 3.6, AB SCIEX) using the search engine Mascot (Version 2.2, Matrix Science, London, UK), and the International Protein Index (IPI) rat database (vision 3.64, 39871 sequences, http://www.ebi.ac.uk/IPI) was used for peptide and protein identification. Search parameters were set as follows: enzyme = trypsin; allowance = up to one missed cleavage; peptide mass tolerance = 100 ppm, fragment mass tolerance = 0.4 Da, fixed
modification = carbamidomethylation (Cys), and variable modification = oxidation (at Met). General protein identification was based on two or more peptides whose ion scores surpassed the statistical threshold ($p < 0.05$). The identified proteins were then matched to specific processes or functions by searching the Gene Ontology database (http://www.geneontology.org/).

Western blotting

Western blotting was introduced to evaluate synaptosomal isolation by the three grinding methods and to confirm the differential expression of two proteins of interest, clathrin light chain A and B, by their respective antibodies. Ten micrograms of protein from the synaptosome fractions prepared were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto a polyvinylidifluoridine membrane. The membrane was blocked with 5% (w/v) skimmed milk solution for 1 h, and then incubated overnight with a primary antibody, mouse anti-syntaxin monoclonal antibody (Stressgen Bioreagents, Victoria, BC, Canada, dilution 1:1000), rabbit anti-clathrin light chain A/B (Clta/Cltb) polyclonal antibodies (Santa Cruz Biotechnology, CA, USA, dilution 1:200), rabbit anti-tubulin β polyclonal antibody (Bioworld Technology, Louis Park, MN, USA, dilution 1:1000, as protein loading control) in the skimmed milk solution at 4°C. After the membrane was washed with Tris–buffer saline with 0.05% Tween-20 (TBST) (150 mM NaCl, 0.05% Tween-20, 10 mM Tris–HCl, pH 7.5), anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (dilution 1:5000) was added to the skimmed milk solution, and the membrane was incubated for 1 h at 37°C. The membrane was washed with TBST, and the blot was developed with ECL reagents. The chemiluminescence signal was imaged using a ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). The data were analyzed with Bio-Rad Quantity One software (Bio-Rad).

Statistical analysis

Using SPSS software, data from the SP test were analyzed by repeated measurement ANOVAs on an experimental group (CMS, control) and time point (baseline, week 1, 2, 3, 4) basis. To detect significant differences between the experimental groups and time points, multivariate analysis of variance (MANOVAs) and Bonferroni post hoc tests were used. The data from Western blotting of Cita and Cltb expression were compared using Student’s t-tests. A $p$-value of $<0.05$ was considered to be statistically significant. Statistics were presented as means ± SE.

RESULTS

Evaluation of three classic grindings in hippocampal synaptosome preparation

Synaptosomal fractions were prepared in parallel by three methods (LNG, HG and MG), characterized by TEM, and differentiated by Western blotting (Fig. 1). Comparative TEM examination demonstrated that the HG and MG methods resulted in more representative structures than the LNG method (Fig. 1A). Western blotting indicated that synaptosomal purity levels in the HG and MG methods were not significantly different, but both were approximately 1.7 times higher than that found in the LNG method ($p < 0.05$). The results demonstrated that the HG and MG methods had a similar effect, whereas both were superior to the LNG method.

CMS model of depression

The CMS rat model employed in this study is one of the most widely-used animal models for inducing depression-like behavior. Repeated measurement ANOVAs displayed no significant differences in water intake.

With respect to sucrose intake, the impact of the experimental group was considered to be significant ($F(1,56) = 21.89, p < 0.0001$), as well as the time point ($F(4,224) = 5.165, p < 0.005$). Bonferroni post hoc tests indicated that following 4 weeks of CMS, the CMS group consumed significantly less sucrose than its respective baseline value ($p < 0.005$). MANOVAs indicated that for both the following 3- and 4-week time periods of CMS, the CMS group consumed less sucrose than the control.
group \( (F(1,56) = 9.099, \ p < 0.005, \text{ and } F(1,56) = 32.105, \ p < 0.0001, \text{ respectively; Fig. 2A}) \).

For the SP test, the impact of the experimental group was significant \( (F(1,56) = 5.922, \ p < 0.05) \), as well as that of the time point \( (F(4,224) = 6.382, \ p < 0.0001) \). Subsequently, Bonferroni post hoc tests indicated that following 4 weeks of CMS, the CMS group consumed significantly less sucrose than its respective baseline value \( (p < 0.0005) \). MANOVAs analysis indicated that following 4 weeks of CMS, the CMS group consumed less sucrose than control \( (F(1,56) = 31.166, \ p < 0.0001; \text{ Fig. 2B}) \). These results indicate that the CMS significantly reduced sucrose intake and preference; therefore, the classic CMS rat model of depression was established.

### Differential analysis of synaptosomes

To explore the molecular mechanism underlying the dysfunctional synaptic transmission and plasticity in MDD, 2DE-based proteomics was utilized to profile differentially expressed proteins in the CMS and control groups. As shown in Fig. 3, approximately 1580 protein spots were detected by silver staining in a single 2DE map. By differential analysis with PDQuest software, 22 spots were differentially expressed, of which 5 were upregulated while 17 were downregulated in the CMS group as compared with the control group.

These 22 spots were then validated by MALDI-TOF/TOF analysis. The MS and MS/MS data were queried using the search algorithm GPS 3.6 (mascot 2.2) against the IPI rat database. Proteins were identified based on a number of criteria including the MW, pi and MASCOT score (Table 2). Nineteen differentially expressed proteins were successfully validated, of which 5 were upregulated and 14 were downregulated in the CMS group as compared to the control group. In these identified proteins, Atp6v1e1, Syn2, Hsp90b1, Hsp91, Dynaj1, Pdia3, Gsn, Actn4, Ina, Pgk1, Aldoc and Acp6 were all downregulated in the CMS group. Furthermore, Nsf, Cita, Cltb and Tpi1 were upregulated in the CMS group. Here it should be noted that of the Dnm1 identified in our study, isoform 2 and 3 of dynamin-1 were downregulated while isoform 5 of dynamin-1 was upregulated in the CMS group.

A comprehensive search was undertaken to determine the subsynaptic location of these 19 validated proteins. There was a high degree of overlap in localization, as 11 proteins localized to the presynaptic terminal and 17 proteins localized to the postsynaptic terminal. Interestingly, the proteins involved in synaptic exo/endo-cytosis localized to both the presynaptic and postsynaptic terminals. Through a literature search, many of these proteins have been reported in different disease states that can affect mood states such as schizophrenia and Alzheimer’s disease. As shown in Table 3. Fifteen proteins have been implicated in these two mental disorders (4 in schizophrenia, 11 in Alzheimer’s disease) and 7 in other mental illnesses (e.g., Down’s syndrome, Huntington’s disease). Furthermore, 4 proteins have been found to be differentially expressed in depressed human patients, and 10 proteins in animal models of depression. The molecular functions of these proteins...
<table>
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<tr>
<th>ID</th>
<th>Accession No.</th>
<th>Accession No.</th>
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<th>Gene name</th>
<th>Protein score</th>
<th>Protein score CI%</th>
<th>Theoretical molecular weight (Da)/pI</th>
<th>Protein abundance ratio (CMS/CON)</th>
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<td>Q6PCU2</td>
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<td><strong>3</strong></td>
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<td>Q9QUL6</td>
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<td>138</td>
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<td>Alpha-internexin</td>
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were classified into five groups according to the GO database and surveys of relevant literature, namely (i) synaptic exocytosis, (ii) synaptic endocytosis, (iii) molecular chaperoning, (iv) cytoskeleton, and (v) energy metabolism (Table 2).

The expression levels of Clta and Cltb were further validated by Western blotting as shown in Fig. 4. The expression levels of Clta and Cltb were significantly increased in the CMS group (fold change = 1.58, \( p < 0.005 \); and fold change = 2.01, \( p < 0.005 \), respectively). These results were consistent with the 2-D electrophoretic findings.

DISCUSSION

The classical monoamine-deficiency hypothesis of MDD initially derived from the therapeutic action of antidepressants. In general, antidepressants have two pharmacologic actions. One, produced by TCA’s, is to increase the synaptic levels of 5-HT and NA by blocking their reuptake. Another, produced by MAOI’s, is to raise intracellular levels of the two neurotransmitters in presynaptic neurons through inhibition of the enzyme monoamine oxidase (Belmaker and Agam, 2008). Moreover, an increased synthesis of monoamine neurotransmitters was previously reported in presynaptic neurons of a chronic-stressed animal model (Anisman et al., 2008). Holm et al. also discovered a reduction in GABA release in a depressed rat model (Holm et al., 2011). Together, these studies show perturbations across the release of multiple neurotransmitters.

Neurotransmitter release is primarily regulated by presynaptic exo/endocytosis (Sudhof, 2004). Recently, LTD has been shown to be facilitated in the hippocampus of a CMS rat model (Holderbach et al., 2007). It is well-established that the facilitation of LTD mainly relies on postsynaptic endocytosis (Wang and Linden, 2000). Taken together, abnormalities in synaptic exo/endocytosis may play a substantive role in MDD pathogenesis.

Therefore, a well-established procedure was used to purify hippocampal synaptosomes to investigate potential abnormalities. Synaptosomes are subcellular membranous structures from the neuronal terminals produced by in vitro subcellular fractionation of brain tissue. Shearing forces lead to the severing of neuronal terminals, and the synaptosomes are generated by subsequent resealing of the membranes. Synaptosomes are then isolated from the homogenate by differential and density-gradient centrifugation. In routine synaptosome preparation, shearing forces are typically produced by some type of tissue grinding procedure. The classical methods include LNG, HG and MG. Aiming to select an optimal strategy, the effect of these different grinding methods on the preparation of rat hippocampal synaptosomes was assessed. Synaptosomal fractions were prepared in parallel by the three methods, characterized by TEM, and differentiated by Western blotting (Fig. 1). Fig. 1A shows that the synaptosomal fractions typically contain the complete presynaptic terminal (including mitochondria and synaptic vesicles.
(SVs) and portions of the postsynaptic side (including the postsynaptic membrane and postsynaptic density). However, the proper synaptosomes are the membrane-bound structures encompassing the SVs, which contain neurotransmitters. Mitochondria are also usually enclosed within these membranes, and the thickened portion of the postsynaptic density is often still attached.

Comparative TEM examination demonstrated that the HG and MG methods resulted in more representative structures than the LNG method (Fig. 1A). Western blotting indicated that synaptosomal purity levels in the HG and MG methods were not significantly different, but both were higher than that found in the LNG method. The LNG method may be more disruptive to tissue structures, thereby affecting synaptosomal enrichment during density-gradient centrifugation. As to the other two methods, the HG method is usually laborious and time-consuming, and thus its applicability to processing a large number of samples is limited and impractical. Therefore, the MG method was selected for synaptosome preparation in the following proteomic analysis.

A 2DE-MS approach was then undertaken that identified 19 differentially expressed synaptic proteins. Interestingly, several synaptic endocytosis- (Clta, Cltb, and Dnm1) and exocytosis-associated proteins (Atp6v1e1, Syn2, and Nsf) were found to be dysregulated in the CMS group.

According to literature on disease relevance, some of these exo/endocytosis-associated proteins have also been found to be differentially expressed in hippocampal protein research on other depressed models, e.g., down-regulation of vacuolar-type H+ ATPase (V-ATPase) in social defeat stress model (Carboni et al., 2006), down-regulation of Syn2 in a learned helplessness model (Mallei et al., 2011), and dysregulation of Nsf and Dnm1 in a gene–environment interaction model (Mallei et al., 2008), (Table 3). In addition, Syn2 was reduced in the

Table 3. Differentially expressed proteins related to mental disorders

<table>
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<th>Gene</th>
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<th>Related to other mental disorders</th>
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<tr>
<td>Atp6v1e1</td>
<td>Social defeat rat model (Carboni et al., 2006), patient with major depression (Beasley et al., 2006)</td>
<td>Alzheimer’s disease (Haass et al., 1995; Lee et al., 2010)</td>
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<td>Syn2</td>
<td>Electroconvulsive therapy rat model (Elfving et al., 2008), learned helplessness rat model (Mallei et al., 2011), maternal separation rat model (Marais et al., 2009), patient with bipolar disorder (Vawter et al., 2002)</td>
<td>Schizophrenia (Imai et al., 2001; Saviouk et al., 2007)</td>
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<td>Nsf</td>
<td>Gene–environment interaction rat model (Mallei et al., 2008)</td>
<td>Schizophrenia (Spillmann et al., 2008), Huntington’s disease (Morton et al., 2001)</td>
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<tr>
<td>Clta</td>
<td>Gene–environment interaction rat model (Mallei et al., 2008), CMS rat model (Kedacka-Krok et al., 2010)</td>
<td>Alzheimer’s disease (Nakamura et al., 1994a), Pick’s disease (Nakamura et al., 1994b)</td>
</tr>
<tr>
<td>Cltb</td>
<td>Patient with bipolar disorder (Kakiuchi et al., 2007), suicide with major depression (Bown et al., 2000)</td>
<td>Alzheimer’s disease (Nakamura et al., 1994a), schizophrenia (Vercauteren et al., 2007)</td>
</tr>
<tr>
<td>Dnm1</td>
<td>Maternal separation rat model (Marais et al., 2009)</td>
<td>Alzheimer disease (Kelly et al., 2005), nicotine dependence (Xu et al., 2009)</td>
</tr>
<tr>
<td>Hsp90b1</td>
<td>Patient with bipolar disorder (Kakiuchi et al., 2007), suicide with major depression (Bown et al., 2000)</td>
<td>Alzheimer’s disease (Imaizumi et al., 2001), Morphine dependence (Ammon et al., 2003)</td>
</tr>
<tr>
<td>Hsp1</td>
<td>Maternal separation rat model (Marais et al., 2009)</td>
<td>Alzheimer’s disease (Kim et al., 2000), Creutzfeldt–Jakob disease (Yoo et al., 2002)</td>
</tr>
<tr>
<td>Dna1</td>
<td>Maternal separation rat model (Marais et al., 2009)</td>
<td>Alzheimer’s disease (Dickson et al., 2005)</td>
</tr>
<tr>
<td>Pdia3</td>
<td>Maternal separation rat model (Marais et al., 2009)</td>
<td>Alzheimer’s disease (Guix et al., 2009)</td>
</tr>
<tr>
<td>Gsn</td>
<td>Maternal separation rat model (Marais et al., 2009)</td>
<td>Mental retardation (Sugie et al., 1989), Down’s syndrome (Labudova et al., 1999)</td>
</tr>
<tr>
<td>Actn4</td>
<td>Social defeat rat model (Carboni et al., 2006)</td>
<td>Alzheimer’s disease (Opii et al., 2008), schizophrenia (Martins-de-Souza et al., 2009)</td>
</tr>
<tr>
<td>Ina</td>
<td>Maternal separation rat model (Marais et al., 2009), social defeat rat model (Carboni et al., 2006), patient with major depression and bipolar disorder (Johnston-Wilson et al., 2000)</td>
<td></td>
</tr>
</tbody>
</table>
postmortem hippocampus of bipolar disorder patients (Vawter et al., 2002), and in turn, its mRNA level in the hippocampus of electroconvulsive therapeutic rat was significantly increased (Elfving et al., 2008). It is worth noting that the dysregulation of Clta and Cltb has not been reported in any previous MDD studies. This novel finding provides a new direction for applied research of potential therapeutic targets for MDD. Further, the biological functions and disease relevance of these altered proteins are detailed below. As visually illustrated in Fig. 5 perturbations in these proteins could result in the SV cycle disruption and LTD facilitation at synapses.

Endocytosis-associated proteins

Clta and Cltb, the two light chains of clathrin, regulate the formation of the clathrin lattice. Notably, the clathrin lattice mediates the endocytosis of SVs at synapses (Granseth et al., 2006). Likewise, the GTPase Dnm1 is essential to endocytic SV fission at the presynaptic plasma membrane (Liu et al., 2006) (Fig. 5A). In Dnm1-knockout mice, SV endocytosis has been found to be severely impaired during strong exogenous stimulation (Ferguson et al., 2007). Taken together, the dysregulation of these three proteins (Clta, Cltb, and Dnm1) potentially creates an obstacle to proper SV endocytosis, thereby negatively affecting the rapid clearance of neurotransmitter release sites, the subsequent priming of SVs, and refilling of the release-ready SV pool (Kawasaki et al., 2011).

Thus far, the relationship between SV endocytosis and MDD has not been a subject of published research, and may be worth further investigation in light of the aforementioned evidence. LTD requires clathrin-mediated glutamate receptor subunit 2 (GluR2)-containing AMPARs endocytosis that is also dependent on Dnm1 (Carroll et al., 1999; Wang and Linden, 2000) (Fig. 5B). The up-regulation of Clta and Cltb found in the CMS group may lead to an increase in this endocytosis. The abnormal expression of Dnm1 may also result in the difficult fission

![Fig. 5. Functional roles of synaptic exo/endocytosis-associated proteins. (A) SV cycle at the presynapse. V-ATPase provides energy by ATP hydrolysis to pump protons into the SV, which forms a proton gradient across the vesicular membrane; transmitters are transported through specific channels coupled to this gradient. SVs are tethered to the cytoskeleton by Syn2; its auto-phosphorylation releases vesicles from the cytoskeleton. The SNARE complex creates a pore in the plasma membrane to allow neurotransmitter release into the synaptic cleft, and Nsf disassembles the SNARE complex. SVs are recycled through endocytosis, which is mediated by clathrin formation coated along the vesicular membrane. The GTPase Dnm1 triggers SV fission from the presynaptic membrane. (B) The formation of LTD at the postsynapse. The GluR2-containing synaptic AMPARs internalized by clathrin-mediated endocytosis that underlie LTD. Dnm1 is involved in the fission of the endocytic AMPARs from the postsynaptic membrane. AMPARs may be degraded or returned to the membrane surface in a Nsf-dependent manner. The association between Nsf and GluR2 maintains AMPARs at the postsynaptic membrane.](image-url)
of the endocytic AMPARs from the postsynaptic plasma membrane, thus blocking the recycling of such receptors. Accordingly, the perturbations in these endocytosis-associated proteins (Clta, Cltb, and Dnm1) may be involved in the facilitated LTD mechanism in the hippocampus of CMS rats (Holderbach et al., 2007).

**CONCLUSION**

In conclusion, the CMS rat hippocampal synaptosome was analyzed using a comparative proteomic approach. The CMS-induced dysregulation of several synaptic proteins involved in exo/endocytosis may interfere with synaptic neurotransmitter release, SV retrieval and synaptic LTD expression. The differentially-expressed proteins found here offers researchers new information in deciphering the abnormal synaptic transmission and plasticity found in MDD.

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**REFERENCES**


To some extent, Nsf also plays an important role in the exocytotic insertion of the AMPARs in postsynapses (Carroll et al., 2001). More importantly, the association between Nsf and GluR2 may maintain the AMPARs in the postsynaptic plasma membrane, and inhibit the GluR2–Nsf interaction which would increase clathrin-mediated endocytosis and obstruct LTD generation (Collingridge et al., 2004). Herein, the upregulation of Nsf would increase the expression of GluR2 in the postsynaptic plasma membrane, which is consistent with the results found by Huang et al. through Nsf overexpression (Huang et al., 2005). This event might be a compensatory response to LTD facilitation.

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