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Proteomic Analysis of Rat Prefrontal Cortex after Chronic Lithium Treatment

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Abstract

Although lithium is widely used to treat Bipolar disorder (BD), its therapeutic role in BD is unclear. To gain insights into its mechanism of action we have used proteomic analysis to identify differentially expressed proteins in rat Prefrontal cortex (PFC), a region specifically affected in BD, after six weeks of lithium treatment. Proteins from control and lithium treated rat PFCs were separated by 2 Dimensional - Differential In-Gel Electrophoresis (2D-DIGE) and identified by mass spectrometry. Of the 2198 protein spots resolved, the abundance of 19 proteins was found to be significantly altered in the lithium treated group (with the levels of 5 proteins increasing and those of 14 decreasing). The levels of two protein spots exhibiting significant alteration after chronic lithium exposure were verified by Western blot analysis of rat PFC extracts. The 19 identified proteins represent novel targets for lithium action and participate in diverse functions that converge on a biological network that is specifically related to brain cell survival, prevention of neurodegeneration, and/or suppression of hyperactivity related signaling pathways. The identification of these targets should facilitate a better understanding of lithium's overall effect on mood control.

Keywords: 2D-DIGE; Bipolar disorder; Chronic lithium; Prefrontal cortex; Proteomics

Abbreviations: 2D-DIGE: 2-Dimensional Differential In-Gel Electrophoresis; BD: Bipolar Disorder; IEF: Isoelectric Focusing; IPA: Ingenuity Pathway Analysis; PFC: Prefrontal Cortex; PTM: Post Translational Modification

Introduction

Bipolar Disorder (BD) is a devastating psychiatric disorder affecting ~2% of the world population [1]. Lithium is the most widely used drug for the treatment of BD [2-4] but the precise mechanism of its action remains an area of intense research [5]. Much attention has been focused on lithium's inhibition of inositol monophosphatase-1 and inositol polyphosphate1-phosphatase, both of which lead to the accumulation of inositol 1-phosphates and reduction in *myo*-inositol levels, thus depleting the pool of cellular inositol triphosphate, a critical second messenger generated from membrane phosphoinositides [5-8]. Apart from its effects on the brain inositol-signaling pathway, lithium affects protein kinase B (Akt)/glycogen synthase kinase 3 (Gsk3) signaling cascades [2,6,7,9-12] which may have an effect on neurotropic factors [13,14], cellular metabolism, cytoskeletal organization, and regulation of gene expression [6,10,13-18]. Lithium also modulates a number of signaling pathways involved in neurotransmission, circadian rhythm and arachidonic acid metabolism [19-21], and may prevent neurodegeneration in animal models of Alzheimer's disease (AD), Parkinson's syndrome, Huntington's disease and spinal cord injury [22,23]. These diverse functions suggest a pivotal role for lithium in neural protein regulation in the prefrontal cortex (PFC), a region linked to the pathophysiology of BD [24-27].

Many of the studies on chronic lithium treatment are based on microarray analyses which reflect changes in mRNA levels that may not necessarily reflect quantitative changes in protein levels [28-31]. In this study, we have used whole rat brain PFC to identify novel proteins employing a 2D-DIGE technique and mass spectrometry analysis, after six weeks of chronic lithium treatment under therapeutically relevant conditions. Analysis of these novel proteins and determination of their role in metabolic pathways will enhance our understanding of the therapeutic and neuroprotective actions of lithium.

Materials and Methods

Animal treatment

Male Sprague-Dawley rats (250-300 gm), three per group for 2D-DIGE analysis and four per group for Western blot analysis, were used. Rats in the lithium group were fed standard rat-chow containing 2 gm/kg of lithium carbonate and were provided 0.9% saline to minimize the electrochemical imbalance caused by the diuretic properties of lithium. All rats had free access to food and water. At the end of 6 weeks, the rats were decapitated under deep CO_2 anesthesia. The brain was rapidly dissected on ice and the PFC was isolated and immediately frozen in dry ice and stored in -70°C. All methods using rats were

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approved by the VA Animal Care Committee, which follows the guidelines set forth by the National Institutes of Health, Washington, DC, USA.

2D-DIGE

Rat brain PFC samples were shipped in dry ice to Applied Biomics (Hayward, CA) for 2D-DIGE analysis. Samples were homogenized by sonication at 4°C in 2D-lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS), followed by shaking for 30 minutes at room temperature in the dark. The samples were then centrifuged for 30 min at 10,000 × g at 4°C and the protein content in the clear homogenate was measured using Bio-Rad PCA reagent. Three similar gels were run containing controls (Cy3), lithium treated extracts (Cy5) and internal standard samples (Cy2) for reliable comparison and quantification of samples.

Cyanine dye labeling of samples

30 µg of protein from control and lithium treated samples were labeled with cyanine dyes, Cy3 and Cy5, respectively, with 1.0 µl of diluted CyDyes each and kept in dark on ice for 30 min. For internal standards, equal amounts of control and lithium treated protein samples were mixed and labeled with Cy2. To enable comparative quantification, this mixture was run on every gel to normalize gel to gel variation and to help align all protein spots across different gels. The labeling reaction was stopped by adding 1.0 µl of 10 mM Lysine to each sample, and then incubated in the dark on ice for an additional 15 min. All samples were then mixed together. The 2X 2-D Sample buffer (8 M urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalytes and trace amount of bromophenol blue), 100 µl DeStreak solution and Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes and trace amount of bromophenol blue) were added to the labeling mix for a total volume of 250 µl. Samples were mixed well before loading onto the strip holder.

IEF and SDS-PAGE

After loading the samples, IEF (pH 3-10 Linear) was performed, following the protocol of Amersham BioSciences (Piscataway, NJ). Upon completion of IEF, the IPG strips (Invitrogen, Carlsbad, CA) were incubated in freshly made equilibration buffer-1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, with trace amount of bromophenol blue and 10 mg/ml DTT) for 15 minutes with gentle shaking. Later, the strips were rinsed in freshly made equilibration buffer-2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 45 mg/ml DTT) for 10 minutes with gentle shaking. The IPG strips were then rinsed in the SDS-gel running buffer, transferred to 12% SDS-gels and were run using standard conditions at 15°C until the dye front ran out of the gels. The gels were scanned immediately using Typhoon TRIO (GE Healthcare, Buckinghamshire, UK). The scanned images were analyzed by ImageQuant software (Version 6.0, GE Healthcare). Image analysis was performed by the DeCyder software (GE Healthcare) to quantitate the changes in protein levels and to determine statistical significance.

Protein identification

Protein spots significantly different from control derived protein spots were picked by Ettan Spot Picker (GE Healthcare) based on analysis by the DeCyder software (version 6.0). The spots of interest were washed four times with 25 mM ammonium bicarbonate in 50% acetonitrile and then digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). Tryptic peptides were desalted from Zip-tip C18 (Millipore) columns using 0.5 µl of matrix solution (5 mg/ml of cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate) and spotted on AB SCIEX MALDI plate (Opti-TOF™ 384 Well Insert). MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an AB SCIEX TOF/TOF™ 5800 System (AB SCIEX). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions). Average value per spot of protein was calculated across triplicate gels and spots that demonstrated > 1.2 fold difference and p-values of < 0.05 (ANOVA) were chosen for identification by mass spectrometry. Confidence intervals and corrected p-values were calculated using a Rank Wilcoxson test. Significance was assessed using a 0.5 threshold of the false discovery rate (FDR). The protein spots were subjected to tryptic digestion followed by MALDI-TOF/TOF analysis. The peptide fragments were identified using the MASCOT search engine. Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage allowed in the search parameters. Both the resulting peptide masses and the associated fragmentation spectra were submitted to GPS-Explorer workstation equipped with MASCOT search engine (Matrix science) to search the National Center for Biotechnology Information non-redundant (NCBInr) database. Candidates with either protein or ion scores of more than 95 % C.I. (Confidence Interval) were considered significant. Detailed results of the aforementioned proteomic analyses are provided in the Supplementary File.

Western-blot analysis

Peptides corresponding to Synuclein A (Snca) and synaptosomal-associated 25 kDa protein (Snap25) were identified by 2D-DIGE to be significantly affected by chronic lithium treatment and exhibited increased and decreased levels, respectively. The levels of these proteins were quantified by Western blotting. PFC samples from four controls and four lithium fed rats were used. Protein was extracted with 2 volumes (wt/vol) of 4 mM HEPES buffer containing 320 mM sucrose and protease inhibitors using a glass homogenizer and motorized Teflon pestle. The samples were centrifuged at 9,000 × rpm for 15 min at 4°C in a Tomy MTX-150 centrifuge. Supernatant protein was quantified using Pierce's BCA protein estimation kit. 50 µg of protein were loaded per well in 12% SDS-polyacrylamide gel, resolved and transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with monoclonal antibodies from Abcam (Cambridge, MA, USA) against Snca (ab1903) or Snap25 (ab24737) and were visualized using ECL chemi luminescence reagents (GE Healthcare). β-Actin was used as control and was detected using the monoclonal antibody MAB1501 from Chemicon International (Temecula, CA, USA). Band intensities were determined using the ImageJ software (http://rsb.info.nih.gov/ij/) and were normalized to corresponding β-actin levels. The levels of these proteins in lithium-fed rats (mean ± S.E.) were expressed relative to that observed in control rats, taken as 100%. All protein band intensities were averaged from three runs.

Results

Identification of differentially expressed proteins by 2D-DIGE after chronic lithium treatment

Total proteins extracted from PFC of three control and three lithium fed rats were analyzed by 2D-DIGE. Of the 2198 protein spots resolved, 19 protein spots were identified to be significantly altered (> 1.2 fold; $p < 0.05$) in the lithium treated group as compared to the control group - 5 peptides exhibited increased levels whereas 14 exhibited decreased levels; the latter included two Map2 forms, one of which was phosphorylated. A representative 2-D gel with the identified protein spots is shown in Figure 1.

Identification and characterization of the differentially expressed proteins in rat PFC after lithium treatment

The 19 differentially expressed proteins, inferred from the protein spots, were analyzed using IPA (Ingenuity Pathway Analysis; Ingenuity Systems, Mountain View, CA) analysis. When grouped according to their biological function (Table 1, Figure 2), many of the 19 differentially expressed proteins were found to be involved in synaptic functions, cellular energy metabolism, signaling processes, and cytoskeletal functions. Lithium-induced changes in protein levels are shown in Table 2. Levels of Efemp2 and Snap25 were significantly reduced (> 1.7 fold) whereas those of Snca and Hk1 were significantly increased (> 1.3 fold).

Western blot analysis of Snca and Snap 25

Western blot analysis was used to verify the levels of two of the most affected proteins – Snca and Snap 25 – in rat PFC. Snca levels were increased by 41% ($p<0.0001$; n=4), whereas Snap25 levels were decreased by 17% ($p<0.0015$; n=4) in lithium fed rats when compared to levels in control rats (Figure 3). These results are consistent with the 2D-DIGE findings (Table 2).

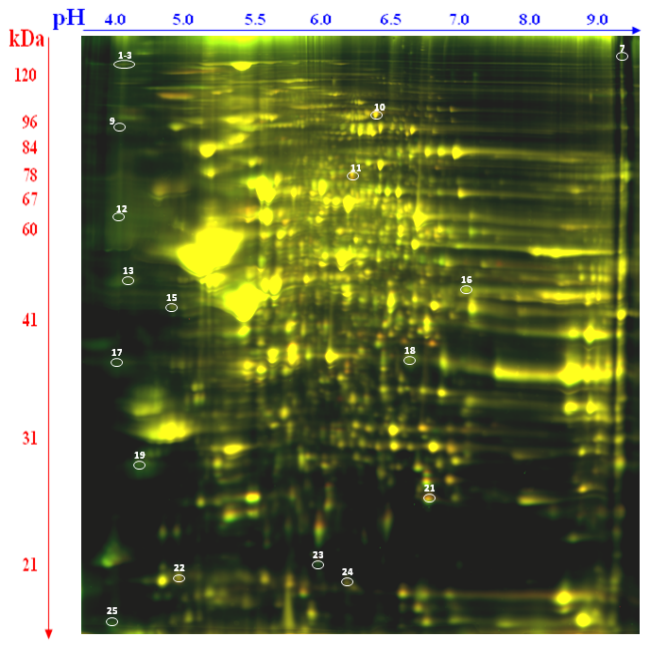

Figure 1: A representative 2D-gel image of Cy5 and Cy3 - labeled PFC proteins. Proteins from control rat PFC were labeled with Cy5 and shown as pseudo-green fluorescence. Lithium-treated rat PFC proteins were labeled with Cy3 and shown as pseudo-red fluorescent spots. In the merged image, shown in the figure, proteins with equal abundance appear as yellow spots, while proteins that are increased or decreased after lithium treatment appear as red or green spots, respectively. The numbered protein spots were identified and are discussed in this paper.

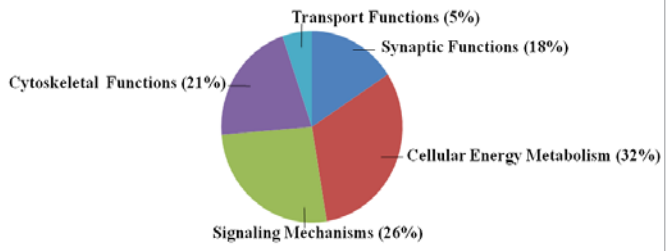

Figure 2: Functional grouping of differentially expressed proteins. The 19 proteins differentially expressed in rat PFC, after chronic lithium treatment, were grouped according to their biological function using Ingenuity Pathway Analysis. The major affected categories include cellular energy metabolism, signaling mechanisms, cytoskeletal functions, synaptic functions and transport functions.

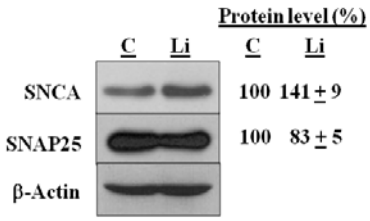

Figure 3: Determination of Snca and Snap25 levels in chronic lithium treated rat PFC. The figure shows results from a representative Western blot experiment. Band intensities for Snca (n = 4) and Snap25 (n = 4) were quantified using the *ImageJ* software and values are expressed relative to corresponding β-actin levels. Values are expressed as percentages (mean ± S.E.) in lithium treated rats compared to control (C) samples set at 100% ($p < 0.05$).

Discussion

Lithium is the drug of choice for the treatment of BD and its clinical efficacy is apparent only after chronic treatment. Little is known about the global changes occurring in brain protein levels after lithium therapy. Our present study represents a model of chronic lithium administration that is clinically and physiologically relevant. A number of studies have examined changes in mRNA levels [28-31] but not changes in protein levels. This work addresses this omission by analyzing changes in protein levels in whole rat PFC, a BD susceptible region [24,26,32], and supports an earlier study that focused specifically on the synapto-neurosomes of the PFC [27].

Using 2D-DIGE and mass spectrometry analysis, we determined the relative levels of 2198 protein spots, 19 of which were significantly different in the PFC of chronic lithium fed rats when compared to controls. Levels of five protein spots were increased and 14 were decreased (the latter included two Map2 polypeptides, one of which was phosphorylated). The 19 identified proteins classified according to their known functionality (based on IPA analysis; Table 1), are involved in biological processes that are relevant to the therapeutic action of lithium, such as triggering anti-apoptotic mechanisms, modulating synaptic function, regulating cell-cell signaling, and remodeling the cytoskeleton architecture. The changes in the levels of the above proteins, coupled with their known functions, may help explain their

	Protein	Location	Biological function
Synaptic	**Snap25** (Synaptosomal-associated 25kD protein)	Cytoplasm, plasma membrane, cell junction synaptosome	Neurotransmitter uptake and secretion; synaptic transmission; synaptic vesicle docking during exocytosis; regulation of insulin secretion.
	Cltb (Clathrin, light chain B)	Plasma membrane	Intracellular protein/receptor transport; vesicle-mediated transport.
	Snca (Synuclein α)	nucleus, cytoplasm, plasma membrane	Integration of presynaptic signaling and membrane trafficking; regulation of dopamine secretion; negative regulation of mono-oxygenase activity; response to interferon gamma; negative regulation of histone acetylation.
Metabolic	**Hk1** (Hexokinase type-I, chain B)	Mitochondria, cytoplasm	Glycolysis; anti-apoptosis; energy and glucose metabolism.
	Gapdh (Glyceraldehyde-3-phosphate dehydrogenase)	Cytoplasm,nucleus,membrane, perinuclear region	Glucose metabolism; glycolysis; oxidation reduction; apoptosis; energy metabolism.
	Psmb2 (Proteasome [prosome, macropain] subunit β type 2)	Nucleus, cytoplasm, proteasome	Ubiquitin-dependent protein degradation.
	Vars (Valine-tRNA synthetase)	Nucleus	Valine-tRNA aminoacylation.
	Trap1 (TNF receptor-associated protein 1)	Cytoplasm, mitochondria	ATP binding; adaptation to oxidative stress; chaperone activity; protein folding.
Signaling	**Calr** (Calreticulin)	Cytoplasm	Ca^{2+} binding; regulator of transcription; regulator of apoptosis.
	Rpsa (Ribosomal protein SA)	Cellsurface,cytoplasm,nucleoli ribosome	Translational elongation; cell adhesion; structural constituent of ribosome; receptor activity; ribosome binding.
	Gap43* (Growth associated Protein 43)	Plasma membrane, synaptosomes	Calmodulin binding; activation of protein kinase C activity by G-protein coupled receptor protein signaling pathway; nervous system development; glial cell differentiation; axon choice point recognition; growth cone guidance; actin dynamics.
	Nudt3 (Nudix [nucleoside diphosphate Linked moiety X]-type motif 3)	Cytoplasm	diphosphoinositol-polyphosphate diphosphatase activity; cell-cell signaling; turnover of inositol pyrophosphates.
	Oas1 (2'-5' oligoadenylate synthetase 1H)		anti-viral action of interferon.
Cytoskeleton	**Basp1** (Brain abundant, membrane attached signal protein 1)	Plasma Membrane	Calmodulin binding; component of brain lipid raft; neuron-specific regulates neurite outgrowth.
	Map2 (**Microtubule-associated protein 2)	Cytoskeleton, cytoplasm	Negative regulation of microtubule depolymerization; calmodulin binding; structural molecule.
	Cnp (2',3'-Cyclic nucleotide 3' phosphodiesterase)	Cytoplasm	Phosphodiesterase activity; microtubule cytoskeleton organization; synaptic transmission; axonogenesis; RNA metabolism.
	Efemp2	Basement membrane, Extracellular space	Extracellular matrix structural constituent; calcium ion binding; protein binding; transmembrane receptor activity.
Transport	**Ensa** (Endosulfine α)	Cytoplasm	Transport; response to nutrient; receptor binding; ion channel inhibitor activity.

Note: *Gap-43 is localized in the plasma membrane and is also a component of the cytoskeletal structure and synapse.
**two forms of Map2 were identified, one of which was phosphorylated.

Table 1: Functional classification of proteins that are differentially expressed in rat PFC upon chronic lithium treatment.

role in disease states. These proteins, therefore, represent novel targets for lithium action and are potential candidates for treatment of BD.

Altered energy metabolism due to malfunctioning of mitochondria is known to contribute to the pathophysiology of psychiatric disorders [32,33]. Brain hexokinase 1 (Hk1) was found to be significantly up-regulated in lithium fed rat PFC (Table 2). This observation establishes a potential link between the up-regulation of Hk1 and the increase in brain glucose-6-phosphate concentrations, observed by Plenge [34], after lithium treatment in normal rats.

BD patients with single nucleotide polymorphisms (SNPs) in the SNAP25 promoter region exhibit aberrantly increased expression of SNAP25 [35]. Of relevance to this study, increased levels of SNAP25 have been observed in dorsolateral PFC of bipolar I patients from postmortem brain samples [36]. Chronic lithium treatment caused a 1.7 fold decrease in Snap25 levels suggesting a mechanism directed against glutamate-induced excitotoxicity in BD. Levels of Cltb, an integral component of the clathrin coated vesicles, were decreased 1.35 fold by lithium treatment, likely resulting in decreased levels of neurotransmitter receptors as well as to a reduction in the rate of synaptic vesicle exocytosis/recycling. These observations strongly suggest that alterations in synaptic processes may have a potential role

Spot No.	Top ranked protein name	Gene Name	NCBI Accession No.	MW (Da) (pI)	MASCOT Score	*p*-value	Fold change
Proteins with increased levels							
22	Synuclein α	***Snca***	GI9507125	14506.2 (4.74)	279	0.006	1.34
10	Hexokinase Type I, Chain B, rat brain	***Hk1***	GI5542104	102455.2 (6.29)	223	0.00023	1.31
21	Proteasome (prosome, macropain) subunit, β type 2	***Psmb2***	GI34849630	22897.7 (6.96)	176	0.014	1.27
24	Endosulfine α	***Ensa***	GI9624979	13326.7 (6.62)	127	0.016	1.26
11	TNF receptor-associated protein 1	***Trap1***	GI55741837	80410.8 (6.56)	99	0.025	1.26
Proteins with decreased levels							
25	EGF-containing fibulin-like extracellular matrix protein 2	***Efemp2***	GI53733803	44820.3 (4.79)	33	0.046	-1.78
19	Synaptosomal-associated 25kD protein	***Snap25***	GI57114057	23300.2 (4.66)	213	0.034	-1.70
1	2'-5' oligoadenylate synthetase 1H	***Oas1h***	GI57222310	42800.7 (5.38)	39	0.024	-1.49
12	Calreticulin	***Calr***	GI11693172	47965.8 (4.33)	343	0.017	-1.47
2*	Microtubule-associated protein 2	***Map2***	GI547890	202287.6 (4.77)	103	0.007	-1.45
3*	Microtubule-associated protein 2	***Map2***	GI56625	198445.6 (4.76)	226	0.036	-1.38
23	Similar to diphosphoinositol polyphosphate phosphohydrolase	***Nudt3***	GI27704734	19083.6 (6.00)	36	0.0011	-1.38
15	Laminin receptor 1/ Ribosomal protein SA	***Rpsa***	GI8393693	32803.4 (4.8)	204	0.04	-1.36
17	Clathrin, light chain B	***Cltb***	GI16758690	25102.1 (4.56)	269	0.015	-1.35
13	Growth associated protein 43	***Gap43***	GI8393415	23589.3 (4.61)	40	0.035	-1.35
9	Brain abundant, membrane attached signal protein 1	***Basp1***	GI11560135	21777.4 (4.5)	70	0.0097	-1.32
7	Valine t-RNA synthetase	***Vars***	GI484949	66744 (6.19)	42	0.024	-1.32
16	2',3'-cyclic nucleotide 3'-phosphodiesterase	***Cnp***	GI294527	47238.6 (9.03)	48	0.012	-1.29
18	Glyceraldehyde-3-phosphate dehydrogenase	***Gapdh***	GI8393418	35805.2 (8.14)	152	0.0038	-1.27

Note: *Spot 2 represents a phosphorylated form of Map2

Table 2: List of differentially expressed proteins in rat PFC after chronic lithium treatment.

in regulating mood stabilization. Increased levels of Snca (1.34 fold) support this hypothesis. Snca binds to tyrosine hydroxylase, the rate limiting enzyme of dopamine synthesis [37]. Increases in Snca levels brought about by chronic lithium treatment can decrease dopamine levels by inhibiting tyrosine hydroxylase synthesis, possibly explaining the dysregulation of dopamergic neurotransmission observed in BD [38,39].

Lithium caused a significant 1.38 fold decrease in the levels of diphosphoinositol polyphosphate phosphohydrolase (Nudt3/Dipp) which is regulated by cellular Ca^{2+} levels, as well as by receptor-mediated cyclic nucleotides [40] which act as a neurotransmitter and possible Akt suppressor [41]. This implies a potential Lithium>Dipp>Akt signaling mechanism that may affect signaling by the Gsk3/Akt pathway. Ensa is a member of the highly conserved cAMP-regulated phosphoprotein family that interacts directly with Snca, and regulates brain G protein coupled receptors and synaptic functions [42]. Thus an increase in Ensa levels is consistent with an increase in Snca levels observed after chronic lithium treatment.

Levels of cytoskeletal proteins are often altered in schizophrenia and BD [43,44]. Our study reveals that chronic lithium treatment decreases Gap-43, Basp1 and Map2 levels. Gap-43 and Basp1 are abundantly present in the inner surface of neuronal plasma membranes and are members of the growth-associated protein family [45,46]. Basp1 stimulates neurite outgrowth [47], possibly by remodeling the actin

cytoskeleton [48], whereas, lithium inhibits neurite outgrowth induced by nerve growth factors [46]. Thus, the decrease in these proteins by lithium may inhibit neurite outgrowth [49].

The fold changes observed in the levels of the various peptides, after lithium treatment, though statistically significant, is quite small. However, small changes in protein levels can have profound effects on cellular function. In particular, post-translational modifications (PTMs) which may significantly impact protein function tend to result in small changes in protein levels [50,51]. It is also likely that various kinds of PTMs (acetylation, carbonylation, glycosylation, hydroxylation, methylation, nitration, palmitoylation, phosphorylation, sulfation, etc.) may have escaped our detection. Nevertheless, the decrease in Map2 phosphorylation after lithium administration (Table 2) is particularly interesting as Sanchez et al. [52] have observed a decrease in site-specific phosphorylation of MAP2 (Thr^{1620}/Thr^{1623}) by GSK3 with lithium treatment in short-term cultured neurons. Map2 phosphorylation appears to be quite complex involving not only Gsk3, but many protein kinases and protein phosphatases [53]. Changes in Map2 phosphorylation can affect microtubule stability affecting neuronal development. Further verification of the observation that Map2 phosphorylation is decreased after lithium treatment is warranted.

It is well known that aberrant neuronal signaling underlies BD pathophysiology and several psychiatric illnesses [54-57]. However the various interlinking mechanisms are not well known. An interactome generated by IPA analysis linking the 19 proteins whose levels were significantly altered by chronic lithium treatment identified several pathways relevant to BD pathology (data not shown). In particular, Protein kinase C (Pkc) was identified as a convergent point for some of these proteins. Activation of Pkc is one of the biochemical hallmarks of BD [58,59]. Hyperactive Pkc signaling [60] is associated with mania, and treatments with lithium or valproate inhibit its activation [57,61], lending support to our analysis. One of the proteins with significantly decreased levels is SNAP25, a direct target of Pkc activation [62]. Decreased SNAP25 levels could result from inability of Pkc to phosphorylate and stabilize SNAP25. In summary, the targets identified in this study have potential therapeutic relevance in the treatment of BD and, therefore, warrant further detailed analysis of the molecular mechanisms by which these proteins are regulated by chronic lithium.

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