Research Article

Proteomic Identification of Altered Cerebral Proteins in the Complex Regional Pain Syndrome Animal Model

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Received 6 March 2014; Revised 14 August 2014; Accepted 25 August 2014; Published 16 September 2014

Academic Editor: Livio Luongo

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Background. Complex regional pain syndrome (CRPS) is a rare but debilitating pain disorder. Although the exact pathophysiology of CRPS is not fully understood, central and peripheral mechanisms might be involved in the development of this disorder. To reveal the central mechanism of CRPS, we conducted a proteomic analysis of rat cerebrum using the chronic postischemia pain (CPIP) model, a novel experimental model of CRPS. Materials and Methods. After generating the CPIP animal model, we performed a proteomic analysis of the rat cerebrum using a multidimensional protein identification technology, and screened the proteins differentially expressed between the CPIP and control groups. Results. A total of 155 proteins were differentially expressed between the CPIP and control groups: 125 increased and 30 decreased; expressions of proteins related to cell signaling, synaptic plasticity, regulation of cell proliferation, and cytoskeletal formation were increased in the CPIP group. However, proenkephalin A, cereblon, and neuroserpin were decreased in CPIP group. Conclusion. Altered expression of cerebral proteins in the CPIP model indicates cerebral involvement in the pathogenesis of CRPS. Further study is required to elucidate the roles of these proteins in the development and maintenance of CRPS.

1. Introduction

Complex regional pain syndrome (CRPS) is a rare but serious and painful disorder. Although CRPS can occur following a minor injury, such as a sprain or even soft-tissue blunt trauma, severe intractable pain from CRPS can impair the quality of life. Symptoms and signs of CRPS include sensory changes (allodynia/hyperalgesia), vasomotor changes (temperature asymmetry/skin color change or asymmetry), sudomotor changes (edema/sweating change or asymmetry), and motor or trophic changes [1]. Although the exact pathophysiology of CRPS is not fully understood, several pathological mechanisms, including oxidative stress [2], neurogenic inflammation [3], and alteration in the autonomic nervous system [4, 5], are known to play some roles in its development. Also, psychophysical studies show that CRPS patients have distorted body image and have difficulty in recognizing the size or the position of the affected extremity [6]. The patients get worse when they think about moving the body part, even if they do not move it [7]. Mechanical stimulation of the “virtual (unaffected)” limb reflected in the mirror results in allodynia, which suggests that allodynia and paresthesia can be mediated by the brain [8]. Thus, the distorted body representation of CRPS patients can be treated with mirror therapy [9, 10]. Also, the spreading of symptoms and signs of CRPS from the initial site of presentation to another limb is a well-known phenomenon, which may be due to aberrant central regulation of neurogenic inflammation [11]. These findings highlight the contribution of a cortical pain mechanism in patients with CRPS. Moreover, functional imaging studies provide supporting evidence for the important role of the central nervous system in the
pathogenesis of CRPS [12–14], and recent research suggests that changes in cortical structures can contribute to the pathophysiology of CRPS [15].

Thus, the brain seems to play an important role in the development and maintenance of symptoms and signs in patients with CRPS. Some researchers insist that the peripheral changes in CRPS must be understood as a manifestation of changes in the brain [16]. Therefore, we postulated that protein expression would be altered in the CRPS-affected brain. However, there have been no studies on the changes of cerebral protein expression in CRPS. Therefore, to verify our hypothesis, we conducted a proteomic analysis using multidimensional protein identification technology (MudPIT) in a chronic postischemia perfusion (CPIP) rat model, a novel and widely used experimental model of CRPS type 1 [17].

2. Materials and Methods

2.1. Animals. This study was approved by the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital (IACUC number 52-2009-033). Male Sprague-Dawley rats weighing 200–250 g had free access to food and water and were housed individually in cages with soft bedding under a 12 h night/day light cycle at a constant temperature of 20–22 °C and a humidity level of 55–60%. The animals were acclimatized for at least 1 week prior to the CPIP procedure.

2.2. CPIP Model Generation. The CPIP animal model was generated according to previous methods [17]. Briefly, after induction of anesthesia with isoflurane, a tight fitting O-ring (O-ring West, Seattle, WA, USA) with a 5.5 mm internal diameter was applied to the left hind limb of each anesthetized rat just proximal to the left ankle joint for 3 h. The O-ring was then removed from the anesthetized rat, allowing reperfusion of the hind limb (Figure 1). The animals in the control group underwent anesthesia similar to the CPIP animals, but the O-ring was not placed around the hind limb.

2.3. Behavioral Tests. All behavioral tests were performed during the daylight portion of the regulated circadian cycle between 9 a.m. and 3 p.m. To assess the mechanical threshold, the rats were placed in individual plastic cages with wire mesh bottoms. After 20 min acclimatization, calibrated von Frey filaments (Stoelting Co., Wood Dale, IL, USA) with logarithmically increasing stiffness of 0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g were applied to the midplantar surface of the hind paw. The mechanical threshold was assessed using an up-down statistical method [18]. Then, the change in the mechanical threshold (CMT, %) was calculated. The mechanical threshold was examined during the postreperfusion period: 1 h, 4 h, 24 h, 48 h, day 7, and day 21. The CMT was calculated by following equation:

\[
\text{CMT} (%) = \frac{M_{\text{post}} - M_{\text{pre}}}{M_{\text{pre}}} \times 100.
\]  

We used the findings from the neurobehavioral test on day 21 to classify the animals into groups: rats whose CMT was decreased 50% or more after the CPIP procedure were classified as the successful CPIP (A) group. The mechanical
threshold of the animals in the control (C) group was also examined and compared using repeated-measures analysis of variance. All animals were sacrificed 3 weeks after the CPIP procedure for proteomic analysis.

2.4. Proteomic Analysis. The difference in cerebral protein expression between Groups A and C was explored using a MudPIT as follows.

2.4.1. Protein Extraction. A total of six animals (three from Groups A and C) were used for the mass spectrometry analysis. On the day 21, right half of each rat cerebrum was grinded using a mortar in liquid nitrogen. The tissue powder was kept at –80°C. The tissue powder was resolubilized in a small volume of 8 M urea, 100 mM Tris-HCl, pH 8.5, and 1 mM dithiothreitol (DTT) for two hours. The homogenates were sonicated and centrifuged at 100,000 g for 1h. Next, 5 mM DTT was added to the homogenate for 30 min at 37°C and alkylated with 25 mM iodoacetamide for 30 min at 37°C in the dark. The samples were then diluted with 2 M urea and with 50 mM Tris-HCl, pH 8.0, and digested at 37°C overnight with sequence grade trypsin (Promega Co., Fitchburg, MA, USA) diluted 1:50 in 5 mM CaCl₂.

2.4.2. MudPIT. Peptides were separated with an Agilent 1100 series high-performance liquid chromatography (HPLC) pump (Agilent technologies, Santa Clara, CA, USA) connected to a linear quadrupole ion-trap mass spectrometer (MS, LTQ, Thermo-Finnigan, San Jose, CA, USA) using an in-house-built nanoelectrospray ionization interface. To identify peptides, the ion-trap mass spectrometer was operated in a data-dependent MS/MS mode (m/z 400–2000), in which a full MS scan was followed by 10 MS/MS scans and the temperature of the heated capillary was 200°C. MS/MS spectra were generated in the positive ion mode at an electrospray voltage of 2.5 kV and normalized collision energy of 35%. An analytical column-fused (100 μm internal diameter) silica capillary microcolumn (Polymicro technologies, Phoenix, AZ, USA) was pulled to a fine tip using a laser puller and packed with 7 cm of 5 μm C18 reverse-phase resin, which was connected to an internal diameter of 250 μm fused-silica trapping column packed with 2 cm of SCX followed by 2 cm of C18 resin. Each 30 μg peptide mixture was manually loaded onto separate columns using a pressure vessel. A seven-step chromatography run was carried out on each sample and three buffers were used (buffer A: 5% ACN/0.1% formic acid, buffer B: 80% ACN/0.1% formic acid, and buffer C: 5% ACN/0.1% formic acid/500 mM ammonium acetate).

2.4.3. Data Searching and Analysis. Acquired MS/MS spectra were searched against an international protein index “rat v. 3.78 FASTA-format decoy database” downloaded from European Bioinformatics Institute (EBI, http://www.ebi.ac.uk/). The SEQUEST algorithm [19] was used to find the best matching sequences from the database with BioWorks 3.3 (Thermo Fisher Scientific Inc., Rockford, IL, USA) for fully tryptic peptides. The mass of the amino acid cysteine was statically modified by +57 Da and the differential modification search was performed for oxidation (+16 Da on Met). Xcorr values were based on tryptic peptides and charge states following 1.8 for singly charged peptides, 2.5 for doubly charged peptides, 3.5 for triply charged peptides, and 0.08 for ΔCn (DTSelect v. 2.0.39). The analysis of protein fold-change was quantified by an overall spectral counting method comparison of label-free methods for quantifying human proteins [20].

3. Results

3.1. Behavioral Tests. A total of 14 animals (n = 7 per group) were included in the behavioral test. Before the CPIP procedure, there were no differences in the mechanical threshold between the groups. However, Group A exhibited a significant decrease in the mechanical threshold compared to Group C after the CPIP procedure (P < 0.01, Figure 2). The mean differences of CMT (%) in Group A compared to Group C were −41.5, −73.2, −92.3, −98.2, −92.2, and −95.3 after CPIP procedure 1 h, 4 h, day 1, day 2, day 7, and day 21, respectively.

3.2. Differential Protein Expression in the Rat Cerebrum. A total of 454 proteins were differentially expressed between Groups A and C under the criterion of P value <0.1. Among the 454 proteins, we selected those found in the cerebrum of all study animals in either group and excluded “uncharacterized proteins” and “hypothetical proteins.” Finally, we found 155 differentially expressed proteins between Group A and Group C: 125 increased (Table 6) and 30 decreased (Table 7). Specifically, expression of proteins related to cell signaling (Table 1), synaptic plasticity (Table 2), regulation of cell proliferation (Table 3), and cytoskeletal formation (Table 4) was increased in Group A. Also, expression of a group of protein kinases (calmodulin dependent protein kinase II beta M isoform, casein kinase 2, phosphoenolpyruvate carboxykinase 2, mitogen-activated protein kinase 4, protein
### Table 1: Increased cerebral proteins in the chronic post-ischemia pain group; proteins which might be related to cell signaling.

| Number | Symbol | Description                                                                 | P value |
|--------|--------|-----------------------------------------------------------------------------|---------|
| 1      | Kctd12 | Potassium channel tetramerisation domain containing 12                      | 0.004   |
| 2      | Ecsl   | Evolutionarily conserved signaling intermediate in Toll pathway, mitochondrial | 0.004   |
| 3      | Tns1   | Tensin 1                                                                    | 0.004   |
| 4      | Ccbp2  | Chemokine-binding protein 2                                                | 0.004   |
| 5      | Apba1  | Amyloid beta A4 precursor protein-binding family A member 1                 | 0.008   |
| 6      | Tnc    | Tenascin C                                                                  | 0.008   |
| 7      | Rabl2b | RAB, member of RAS oncogene family-like 2                                   | 0.008   |
| 8      | Epha4  | Eph receptor A4                                                             | 0.035   |
| 9      | Rab6a  | Ras-related protein Rab-6A                                                  | 0.038   |
| 10     | Gpr158 | G protein-coupled receptor 158                                              | 0.042   |
| 11     | Anxa2  | Isoform Short of Annexin A2                                                 | 0.043   |
| 12     | Hgs    | Isoform 1 of hepatocyte growth factor-regulated tyrosine kinase substrate   | 0.054   |
| 13     | Prkcd  | Isoform 1 of protein kinase C delta type                                     | 0.060   |
| 14     | Gabarpl2 | Gamma-aminobutyric acid receptor-associated protein-like 2                  | 0.065   |
| 15     | Map2k4 | Dual specificity mitogen-activated protein kinase 4                         | 0.066   |
| 16     | Cacng2 | Voltage-dependent calcium channel gamma-2 subunit                           | 0.083   |
| 17     | Phb2   | Prohibitin-2                                                                | 0.086   |
| 18     | Camk2b | Calmodulin-dependent protein kinase II beta M isoform                        | 0.086   |
| 19     | Anxa5  | Annexin A5                                                                 | 0.093   |
| 20     | Scn2a1 | Sodium channel Nav1.2                                                       | 0.095   |
| 21     | Rab10  | Ras-related protein Rab-10                                                  | 0.097   |

### Table 2: Increased cerebral proteins in the chronic postischemia pain group; proteins which might be related to synaptic plasticity.

| Number | Symbol | Description                                                                 | P value |
|--------|--------|-----------------------------------------------------------------------------|---------|
| 1      | Itpr2  | Inositol 1,4,5-trisphosphate receptor type 2                                 | 0.001   |
| 2      | Kctd12 | Potassium channel tetramerisation domain containing 12                      | 0.004   |
| 3      | Grid2  | Glutamate receptor delta-2 subunit                                          | 0.004   |
| 4      | Baiap3 | BAI1-associated protein 3-like isoform 2                                    | 0.008   |
| 5      | Atad1  | ATPase family, AAA domain containing 1                                      | 0.008   |
| 6      | Pick1  | PRKCA-binding protein                                                       | 0.008   |
| 7      | Nlgn3  | Isoform 1 of Neureilgin-3                                                   | 0.056   |
| 8      | Nucd   | Nuclear migration protein nudC                                              | 0.085   |
| 9      | Camk2b | Calmodulin-dependent protein kinase II beta M isoform                        | 0.086   |

### Table 3: Increased cerebral proteins in the chronic postischemia pain group; proteins which might be related to regulation of cell proliferation.

| Number | Symbol | Description                                                                 | P value |
|--------|--------|-----------------------------------------------------------------------------|---------|
| 1      | Pik3r4 | Phosphoinositide 3-kinase regulatory subunit 4                              | 0.001   |
| 2      | Itpr2  | Inositol 1,4,5-trisphosphate receptor type 2                                 | 0.001   |
| 3      | Anp32b | Acidic leucine-rich nuclear phosphoprotein 32 family member B               | 0.002   |
| 4      | Pkl1   | Serine/threonine-protein kinase                                             | 0.004   |
| 5      | Drg2   | Developmentally regulated GTP binding protein 2-like                        | 0.004   |
| 6      | Dmwd   | Dystrophia myotonica-containing WD repeat motif                              | 0.008   |
| 7      | Acin1  | Apoptotic chromatin condensation inducer 1 protein                          | 0.008   |
| 8      | Pole2  | Polymerase (DNA directed), epsilon 2                                         | 0.008   |
| 9      | Cyld   | Ubiquitin carboxyl-terminal hydrolase                                        | 0.076   |
| 10     | Csnka2  | Casein kinase 2, alpha prime polypeptide                                     | 0.084   |
| 11     | Rab10  | Ras-related protein Rab-10                                                  | 0.097   |
kinase C delta, N-terminal kinase like protein, uridine kinase like 1, serine/threonine protein kinase PLK 1, and phosphoinositide 3 kinase regulatory subunit 4) and calcium-related proteins (inositol 1,4,5-triphosphate receptor type 2, annexin A1, annexin A2, annexin A5, voltage-dependent Ca\(^{2+}\) channel gamma-2 subunit, and voltage-dependent Ca\(^{2+}\) channel beta-3 subunit, and coiled-coil domain-containing protein 47) was also elevated in Group A. However, several proteins were decreased in group A. Specifically, expression of proteins related to cell signaling (Table 5) and metabolism of fatty acid (peroxisomal 3,2-trans-enoyl Co A isomerase, acetyl-CoA acyltransferase 1b, and acetyl-CoA acetyltransferase 2) were decreased. Also, proenkephalin A, protein cereblon, and neuroserpin were decreased in Group A.

### 4. Discussion

In our study, various proteins were differentially expressed in the cerebrum of CPIP animals. Specifically, expressions of proteins related to cell signaling, synaptic plasticity, regulation of cell proliferation, and cytoskeletal formation were increased in Group A. These findings suggest that both functional and structural changes may occur in the cerebrum of CPIP animals, and altered protein expression can be related to the development of CRPS. This is the first study of cerebral protein expression changes in the CPIP rat model.

We also found that inositol 1,4,5-triphosphate (IP3) receptor type 2 and phosphoinositide 3 kinase (PI3K) regulatory subunit were also increased in Group A. IP3 receptor is intracellular calcium release channel and is regulated by calcium and calmodulin (CaM) [21]. And it is known that PI3K is an important mediator of central sensitization in painful inflammatory condition [22], and many tumorous conditions are related to this enzyme [23, 24]. Based on these findings, cerebral overexpression of IP3 receptor type 2 and PI3K can be related to the sustained pain after rat CPIP model.
| Number | Symbol | Description | P value |
|--------|--------|-------------|---------|
| 1      | Itpr2  | Inositol 1,4,5-trisphosphate receptor type 2 | 0.001   |
| 2      | Pik3r4 | Phosphoinositide 3-kinase regulatory subunit 4 | 0.001   |
| 3      | Exoc7  | Exocyst complex component 7 | 0.001   |
| 4      | Rcor2  | REST corepressor 2 | 0.001   |
| 5      | Anp32b | Acidic leucine-rich nuclear phosphoprotein 32 family member B | 0.002   |
| 6      | Qrich2 | Glutamine rich 2-like | 0.002   |
| 7      | Dnah11 | Dynein, axonemal, heavy chain 11 | 0.002   |
| 8      | Plk1   | Serine/threonine-protein kinase PLK1 | 0.004   |
| 9      | Ephx1  | Epoxide hydrolase 1 | 0.004   |
| 10     | Cacnb3 | Voltage-dependent L-type calcium channel subunit beta-3 | 0.004   |
| 11     | Anxa1  | Annexin Al | 0.004   |
| 12     | Tns1   | Tensin 1 | 0.004   |
| 13     | Hdac4  | Histone deacetylase 4 | 0.004   |
| 14     | Osbp17 | Oxysterol binding protein like 7 | 0.004   |
| 15     | Ecsit  | Evolutionarily conserved signaling intermediate in Toll pathway, mitochondrial | 0.004   |
| 16     | Sorbs3 | Sorbin and SH3 domain containing 3, isoform CRA_b | 0.004   |
| 17     | Kcd1l2 | Potassium channel tetramerisation domain containing I2 | 0.004   |
| 18     | Ccbp2 | Chemokine-binding protein 2 | 0.004   |
| 19     | Drg2   | Developmentally regulated GTP binding protein 2-like | 0.004   |
| 20     | Grid2  | Glutamate receptor delta-2 subunit | 0.004   |
| 21     | Safb   | Scaffold attachment factor B1 | 0.008   |
| 22     | Dnm3   | Isoform 1 of Dynamin-3 | 0.008   |
| 23     | Dnajc6 | DnaJ homolog subfamily C member 16 | 0.008   |
| 24     | Snlb2  | Syntrophin, beta 2 | 0.008   |
| 25     | Pnpt1  | Polyribonucleotide nucleotidyltransferase 1 | 0.008   |
| 26     | Eif3g  | Eukaryotic translation initiation factor 3 subunit G | 0.008   |
| 27     | Pole2  | Polymerase (DNA directed), epsilon 2 | 0.008   |
| 28     | Scyl1  | N-terminal kinase-like protein | 0.008   |
| 29     | Atad1  | ATPase family, AAA domain containing | 0.008   |
| 30     | Krt4   | Keratin, type II cytoskeletal 4 | 0.008   |
| 31     | Ctsa   | Protective protein for beta-galactosidase | 0.008   |
| 32     | Abca1  | 5 ATP-binding cassette, subfamily A (ABCI), member 15 | 0.008   |
| 33     | Dmdw   | Dystrophia myotonica-containing WD repeat motif | 0.008   |
| 34     | Biaa3  | BAI1-associated protein 3-like isoform 2 | 0.008   |
| 35     | Znf512b| Uridine kinase-like 1 | 0.008   |
| 36     | Gale   | Gale protein | 0.008   |
| 37     | Pick1  | PRKCA-binding protein | 0.008   |
| 38     | Acin1  | Acin1 protein | 0.008   |
| 39     | Chid1  | Chitinase domain containing 1 | 0.008   |
| 40     | Pcyoxl | Pcyoxl protein | 0.008   |
| 41     | Rabl2b | RAB, member of RAS oncogene family-like 2B | 0.008   |
| 42     | Serpina3k| Serine protease inhibitor A3K | 0.008   |
| 43     | Glg1   | Golgi apparatus protein 1 | 0.008   |
| 44     | Tnc    | Tenascin C | 0.008   |
| 45     | Lysmd1 | LysM and putative peptidoglycan-binding domain-containing protein 1 | 0.008   |
| 46     | Apba1  | Amyloid beta A4 precursor protein-binding family A member 1 | 0.008   |
| 47     | Ckap5  | Cytoskeleton associated protein 5 | 0.038   |
| 48     | Ndufab1| Acyl carrier protein | 0.035   |
| 49     | Epha4  | Eph receptor A4 | 0.035   |
| 50     | Kalrn  | Isoform 2 of Kalirin | 0.035   |
| 51     | Myh14  | Myosin, heavy chain 14 | 0.035   |
| Number | Symbol | Description                                      | P value |
|--------|--------|--------------------------------------------------|---------|
| 52     | Anxa2  | Isoform Short of Annexin A2                     | 0.043   |
| 53     | Ccdc47 | Coiled-coil domain-containing protein 47        | 0.043   |
| 54     | Gpr158 | G protein-coupled receptor 158                  | 0.042   |
| 55     | Cugbp1 | CUGBP Elav-like family member 1                 | 0.041   |
| 56     | Hba2   | Hemoglobin alpha 2 chain                        | 0.040   |
| 57     | Acsl3  | Isoform long of long-chain-fatty-acid-CoA ligase| 0.040   |
| 58     | Rab6a  | Ras-related protein Rab-6A                     | 0.038   |
| 59     | Hbb    | Hemoglobin subunit beta-1                      | 0.048   |
| 60     | Hbb-b1 | Zero beta-1 globin                              | 0.044   |
| 61     | Khsp1  | Far upstream element-binding protein 2          | 0.043   |
| 62     | Scamp5 | Secretory carrier-associated membrane protein 5| 0.048   |
| 63     | Aldh3a2| Fatty aldehyde dehydrogenase                    | 0.049   |
| 64     | Mesdc2 | LDLR chaperone MESD                             | 0.049   |
| 65     | Rab3d  | GTP-binding protein Rab-3D                      | 0.051   |
| 66     | Vps29  | Isoform 2 of vacuolar protein sorting-associated protein 29 | 0.051 |
| 67     | Psm3l  | Psma3 Proteasome subunit alpha type-3           | 0.053   |
| 68     | Hgs    | Isoform 1 of hepatocyte growth factor-regulated tyrosine kinase | 0.054 |
| 69     | Nlgn3  | Isoform 1 of neureilgin-3                      | 0.056   |
| 70     | Cygb   | Cytoglobin                                       | 0.060   |
| 71     | Pcsk2  | Neuroendocrine convertase 2                     | 0.060   |
| 72     | Prkcd  | Isoform 1 of Protein kinase C delta             | 0.060   |
| 73     | Fnbp4  | Formin binding protein 4                        | 0.062   |
| 74     | Eif2s3x| Eukaryotic translation initiation factor 2 subunit 3 | 0.063 |
| 75     | Fermt2 | Fermitin family homolog 2                       | 0.063   |
| 76     | Vps33a | Vacuolar protein sorting-associated protein 33A | 0.063 |
| 77     | Snx3   | Sorting nexin-3                                | 0.063   |
| 78     | Exoc8  | Exocyst complex component 8                     | 0.063   |
| 79     | Thrap3 | Thyroid hormone receptor-associated protein 3   | 0.063   |
| 80     | Ndufa1 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1 | 0.063 |
| 81     | Gabarpl2| Gamma-aminobutyric acid receptor-associated protein-like 2 | 0.065 |
| 82     | Cottl  | Coactosin-like protein                          | 0.065   |
| 83     | Gad1   | Glutamate decarboxylase 1                       | 0.065   |
| 84     | Eh1    | EH domain-containing protein 1                  | 0.066   |
| 85     | Mapk24 | Mitogen-activated protein kinase 4              | 0.063   |
| 86     | Muml  | Murinoglobulin (alpha-1-inhibitor 3)            | 0.070   |
| 87     | Pck2   | Phosphoenolpyruvate carboxykinase 2             | 0.072   |
| 88     | Rps5   | 40S ribosomal protein S5                        | 0.072   |
| 89     | Ap2si  | Adaptor protein complex 2 subunit sigma         | 0.075   |
| 90     | Tpp1   | Tripeptidyl-peptidase 1                         | 0.076   |
| 91     | Cyl1   | Ubiquitin carboxyl-terminal hydrolase           | 0.076   |
| 92     | Nuc    | Nucleolin-like protein                          | 0.079   |
| 93     | Col1a2 | Collagen alpha-2(I) chain                      | 0.079   |
| 94     | Slc6a17| Orphan sodium- and chloride-dependent neurotransmitter transporter NTT4 | 0.079 |
| 95     | Actr10 | Actin-related protein 10 homolog                | 0.080   |
| 96     | Cacng2 | Voltage-dependent calcium channel gamma-2 subunit | 0.083 |
| 97     | Ampd3  | AMP deaminase 3                                 | 0.083   |
| 98     | Eif5b-ps1| Eukaryotic translation initiation factor 5B     | 0.083   |
| 99     | Timm9  | Mitochondrial import inner membrane translocase subunit Tim9 | 0.083 |
| 100    | Eti4   | Enhancer trap locus 4-like                     | 0.083   |
| 101    | Csnk2a2| Casein kinase 2, alpha prime polypeptide        | 0.084   |
| 102    | Cct6a  | Chaperonin containing TCP1 subunit 6a           | 0.084   |
| 103    | Nudc   | Nuclear migration protein nud                   | 0.085   |
Table 6: Continued.

| Number | Symbol | Description                                                                 | P value |
|--------|--------|-----------------------------------------------------------------------------|---------|
| 104    | Ndufa13| NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 13                        | 0.085   |
| 105    | Camk2b | Calmodulin-dependent protein kinase II beta M isoform                        | 0.086   |
| 106    | Clta   | Isoform brain of clathrin light chain A                                     | 0.086   |
| 107    | Asah1  | Acid ceramidase                                                             | 0.086   |
| 108    | Phb2   | Prohibitin-2                                                                | 0.086   |
| 109    | Sod1   | Superoxide dismutase [Cu-Zn]                                                | 0.088   |
| 110    | Ndufs8 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 8                | 0.090   |
| 111    | Slc17a7| Isoform 1 of vesicular glutamate transporter 1                               | 0.091   |
| 112    | Ugp2   | UDP-glucose pyrophosphorylase 2, isoform CRA-b                               | 0.091   |
| 113    | Rala   | Ras-related protein Ras-A                                                   | 0.091   |
| 114    | Anxa5  | Annexin A5                                                                  | 0.093   |
| 115    | Hnph1  | Isoform 1 of heterogeneous nuclear ribonucleoprotein H                       | 0.093   |
| 116    | Stxbp5l| Syntaxin binding protein 5-like                                              | 0.093   |
| 117    | Abcd3  | ATP-binding cassette subfamily D member 3                                   | 0.094   |
| 118    | Farp1  | FERM, RhoGEF (Arhgef), and pleckstrin domain protein 1                      | 0.094   |
| 119    | Leng4  | Leng4 protein                                                                | 0.094   |
| 120    | Scn2a1 | Sodium channel Nav1.2                                                        | 0.095   |
| 121    | Rab10  | Ras-related protein Rab-10                                                  | 0.097   |
| 122    | Aldh7a1| Alpha-aminoisopinic semialdehyde dehydrogenase                              | 0.097   |
| 123    | Cltb   | Isoform Brain of Clathrin light chain B                                     | 0.097   |
| 124    | Phyhipl| Isoform 1 of phytanoyl-CoA hydroxylase-interacting protein-like             | 0.098   |
| 125    | Synpo  | Isoform 1 of synaptotodin                                                   | 0.099   |

Among the 155 proteins expressed differentially, calcium related proteins, including calcium calmodulin kinase II (CaMKII), were increased in group A. Calcium plays a crucial role in many physiological processes, including signal transduction, cell growth, and proliferation. CaMKII is one of the most prominent protein kinases, present in every tissue, but most concentrated in the brain. CaMKII plays various roles, including synthesis and release of neurotransmitter modulation of ion channel activity, synaptic plasticity, learning, and memory [25–28]. Moreover, CaMKII is thought to be important in central sensitization [29–31] and is implicated in central neuropathic pain [31]. LTP is initiated when NMDA receptors allow Ca$^{2+}$ into the postsynaptic neuron, and this Ca$^{2+}$ influx activates CaMKII. LTP in nociceptive spinal pathways shares several features with hyperalgesia, and LTP at synapses constitutes a contemporary cellular model for pain [33, 34]. And it was reported that the overexpression of CaMKII was observed in the dorsal root ganglia of rat model of type 1 diabetes [35], and the inhibition of CaMKII can reverse the chronic inflammatory pain [36]. These findings are consistent with the result of our study. Therefore, overexpression of cerebral CaMKII implicates cerebral involvement in CRPS, and CaMKII can be a target for the prevention and treatment of CRPS.

In our study, we used the CPIP model because CRPS develops after a minor injury without distinguishable nerve lesions. This model is considered a novel animal model of CRPS type 1, in which nerve injury or bone fracture usually does not exist. The previous proteomic studies in neuropathic pain research usually used the nerve ligation model or nerve crush injury model [43, 44]. Since the CPIP model exhibits similar features of human CRPS type 1, our results may have an implication for cerebral involvement in human CRPS. The mechanical threshold was similar at the beginning (day 1) and after 21 days. This is because we took no actions for treatment on the CPIP animals and therefore the initial pain seemed to persist without change. We did not measure the mechanical threshold in the contralateral paws, because it has...
been already known that the mechanical threshold decreases in the contralateral paws of the CPIP animals, and ipsilateral plantar allodynia is known to be the most characteristic feature of the CPIP animals [17]. The CMT in the ipsilateral paw was used for the criterion of the successful establishment of the animal model.

This study had some limitations. First, the differentially expressed cerebral proteins may not be specific to CPIP animals. These proteins may also change in response to peripheral noxious stimuli. However, CPIP animals exhibit many features of human CRPS type 1, and thus our findings can be extrapolated to human CRPS. Second, because of the complexity of protein interactions in many physiologic pathways in the brain, it is still unclear which is the key protein in the development of CRPS.

Third, we performed proteomic analysis only 21 days after CPIP model generation. However, protein expression related to the development and maintenance of CRPS can differ according to the time course. A proper time course to evaluate a possible correlation between pain behavior development and protein modulation may be useful to discriminate protein changes associated with the early inflammation from that one responsible for possible structural or functional alterations (neural sensitization) occurring at central level. Further investigation of the cerebral mechanism of CRPS is required.

5. Conclusion

In conclusion, the cerebral proteome is altered after CPIP injury; many functional and structural changes seem to occur in the cerebrum. These findings support the notion of cerebral involvement in CRPS. Therefore, treatment of CRPS should target not only the periphery, but also the brain.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgment

This study was financially supported by Grant no. 02-2009-038 from the Seoul National University Bundang Hospital.
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