Quantitative Proteomic Analysis of the Central Amygdala in Neuropathic Pain Model Rats

Kersti Karu, Richard S. Swanwick, Amparo Novejarque-Gadea, Ana Antunes-Martins, Benjamin Thomas, Eiji Yoshimi, William Foster, Min Fang, Stephen B. McMahon, David L. H. Bennett, Andrew S. C. Rice, and Kenji Okuse*

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ABSTRACT: Pain and emotional distress have a reciprocal relation. The amygdala has been implicated in emotional processing. The central nucleus of the amygdala (CeA) receives nociceptive information from the dorsal horn of spinal cord and is responsible for the central plasticity in chronic pain. Neuropathic pain is a type of severe chronic pain and can be strongly influenced by emotional components. Plastic changes in the CeA may play a key role in the development or maintenance or both of neuropathic pain. We studied the expression levels of proteins in the CeA of spinal nerve transection (SNT) model rats. Total tissue lysate proteins were separated by two-dimensional-gel electrophoresis (2D-PAGE). Gels from different time points were compared using Progenesis SameSpot software, and the spots with Fold Change greater than 2 were excised for protein identification by mass spectrometry. We identified more than 50 cytosolic proteins as significantly altered in their expression levels in the CeA of SNT rats, and most of these changes have been validated at mRNA levels by qRT-PCR. We also identified more than 40 membrane proteins as notably up- or down-regulated in the CeA of SNT model rats relative to a control using stable isotope dimethyl labeling nano-LC-MS/MS based proteomics and found that one such protein, doublecortin (DCX), a microtubule-associated protein expressed by neuronal precursor cells during development, is specifically localized in the membrane fraction without changes in total amount of the protein. Immunohistochemistry showed that doublecortin is expressed in processes in the CeA of rats 7 and 21 days after SNT surgery, suggesting that doublecortin is one of the proteins that may contribute to the plastic changes, namely, redevelopment or rewiring of neural networks, in the CeA in the neuropathic pain model. These dysregulated proteins may play roles in reciprocal relationships between pain and psychological distress in the amygdala and contribute to central sensitization. Data are available via ProteomeXchange with identifier PXD017473.

KEYWORDS: amygdala, neuropathic pain, proteomic, mass spectrometry, spinal nerve transection, LC-MS/MS, stable isotope dimethyl labeling

INTRODUCTION

Pain has a profound impact on the level of emotional distress, and psychological states play an important role in determining the extent of pain. Thus, the relationship between pain and psychological distress such as depression and anxiety appears to be reciprocal. The amygdala is a structure in the brain located in the anterior inferior temporal lobe and has been implicated in emotional processing. The amygdala can be divided into anatomically and functionally distinct nuclei, namely basal, lateral, medial, and central nucleus. Among them, the central nucleus of amygdala (CeA) serves as the output nucleus for major amygdala functions, while other nuclei are involved in receiving input from the sensory systems, the sense of smell and pheromone-processing, and emotional arousal. The capsular part of the CeA is defined as the nociceptive

amygdala, because it receives nociceptive information from the dorsal horn of spinal cord.

Neuropathic pain is a type of severe chronic pain that is generally refractory to treatment. Although neuropathic pain is defined as pain caused by a lesion or disease of the somatosensory nervous system, it can also be strongly influenced by emotional components. Neuropathic pain is associated with plastic changes in both peripheral and central

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nervous systems.¹ Neural plasticity includes changes in biochemical profile, function, and structure of neurons. The CeA is thought to be one of the key areas responsible for the central plasticity in chronic pain including neuropathic pain, because the CeA integrates nociceptive input from the spinal cord and polymodal information from the thalamus and cortex. In fact, the CeA has been found to exhibit a high degree of plasticity in chronic pain models. The immediate-early gene *c-Fos* and metabotropic glutamate receptors have been found to be upregulated in the CeA in chronic pain conditions.^{2,3} The excitability of CeA neurons has also been found to be increased in rats with neuropathic pain⁴ and arthritic pain^{1,5} conditions. These plastic changes in the CeA may play a key role in the development or maintenance of chronic pain or both; however little is known about the regulation of cell proliferation and differentiation in the amygdala in response to neuropathic pain. Only a few studies have actually examined the amygdala in depth at the proteome level.^{6–8}

Transcriptomics or genetic analysis provides a partial view of the signaling pathways driving neuropathic pain response because of their limited capacity to predict concentrations changes at the protein level or inform about status of protein changes.^{9,10} Mass spectrometry (MS)-based proteomics analysis of the central amygdala proteins that change in response to spinal nerve transection (SNT) may have a large potential to unveil novel signaling mechanisms of clinical relevance.^{9,11} Quantitative proteomics further enable identification of the altered expression of proteins. One of the most utilized approaches in proteomics to identify differential proteins in complex samples is isoelectric focusing two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)-based MS.^{12,13} The silver stained spots between different gels are compared using spot matching software alignments. The differential 2D-PAGE spots are excised and in-gel digested with an enzyme, and the resulting peptides are separated on the LC column prior to mass spectrometry analysis. Another approach is to use two-dimensional difference gel electrophoresis, which requires an extra step to prelabel proteins with fluorescent dyes before isoelectric focusing (2D-DIGE). By use of different dyes on 2D-DIGE, multiple samples can be run and compared on the same gel without using spot matching programs.⁷ These 2D gel based MS methods offer high accuracy fold changes of proteins on the basis of the intensity of the protein spots on the gel. Unfortunately, these proteomics approaches provide only a very incomplete picture with some limitations for understanding of the molecular mechanisms underlying neuropathic pain at the protein level.¹⁴ It has become apparent that 2D gels are almost exclusively restricted to cytosolic proteins with a very low content of membrane proteins such as ion channels and receptors.^{9,10,15} Since many membrane proteins are of low abundance, enrichment of membranes or isolation of specific type of membrane proteins is beneficial for an extensive analysis. One of the approaches is the 1D gel based or gel-free MS strategies, which are used to analyze integral membrane proteins.⁹ These methods are not reliable for quantification on their own; therefore, additional methods for quantification have been developed. Isobaric tags such as iTRAQ have enabled quantitative analysis of samples without relying on 2D gels.¹⁶ Recently more cost-effective methods, namely multiplex peptide stable isotope dimethyl labeling, have been developed.¹⁷ Stable isotope dimethyl labeling does not require introduction of isotope labels at the organism level like SILAC

(stable isotope labeling by amino acids in cell culture)^{18,19} and uses inexpensive reagents.

To capture the global protein changes in the CeA from the SNT model rats, we utilized three proteomics approaches in this work: (a) analysis of the whole cell lysate of the CeA using 2D-PAGE MS, (b) purification of CeA membrane proteins and separation on 1D-SDS-PAGE, followed by excision of the gel bands between 80 and 230 kDa and nano LC-MS/MS analysis of digested peptides, and (c) quantitative mass spectrometry-based proteomics analysis using triplex stable isotope dimethyl labeling of the CeA membrane proteins. We compared membrane proteomes of the CeA from sham-operated rats and rats at days 3, 7, and 21 after SNT surgery.

The 2D gel based proteomic study revealed that more than 50 cytosolic proteins were altered in the CeA of SNT model rats. No significant proteome changes ($F > 4$) were observed in the left- and right-side CeA. Using the 1D-SDS-PAGE approach, we identified neurotransmitter receptors, such as glutamate and GABA receptors, sodium and potassium ion channel proteins, and voltage-dependent ion channels, such as potassium voltage-gated channel subfamily A member 3 and Nav1.2. Using triplex stable isotope dimethyl labeling combined with LC-MS/MS based proteomics, we identified more than 40 membrane proteins as differentially expressed proteins in the CeA of SNT model rats. These proteins may play roles in the reciprocal relationship between pain and psychological distress in the amygdala and contribute to central sensitization. Doublecortin was identified with the highest fold change in all three biological replicates after the SNT surgery. Most of these changes of protein expression were validated by qRT-PCR, Western blotting, and immunohistochemistry.

MATERIALS AND METHODS

Animals, SNT and Sham Surgery, and Behavior Assessment

Two groups of animals were used in this study. The first group (group 1) of animals were adult male Wistar rats (200–300 g) with spinal nerve transection (SNT model rats) produced by tight ligation and transection of the left L5 spinal nerve. Briefly, animals were anesthetized using a mixture of medetomidine hydrochloride (0.25 mg/kg) and ketamine (60 mg/kg) administered in a single intraperitoneal injection. The animals were placed in the prone position, and under sterile conditions a paramedial incision was made to access the left L4–L6 spinal nerves. Approximately one-third of the L6 transverse process was removed. The L5 spinal nerve was identified and carefully dissected free from the adjacent L4 spinal nerve and then tightly ligated using 6–0 silk and transected distally to the suture. The muscle layer was sutured, and the wound was closed with Vicryl 3–0. Mechanical withdrawal thresholds were tested using a Dynamic Plantar Aesthesiometer (Ugo Basile). A maximum cutoff of 50 g was used. The withdrawal threshold is calculated as the average of three consecutive tests with at least 10 min between each test. To measure cold hypersensitivity, we applied a drop of acetone to the plantar hind paw and measured the time that the animal spent licking, shaking, or lifting the paw during the following 2 min. Sham rats underwent a skin incision, muscle dissection, etc., but no injury was made in the spinal nerves. All behavioral tests were blinded to randomization schedule. All animal experiments conformed to the British Home Office regulations and

178 followed International Association for the Study of Pain
179 guidelines for the care and use of animals. The animals were
180 deeply anesthetized and decapitated, and their brains were
181 frozen on dry ice within 3 min of decapitation and stored at
182 -80°C . Animals were culled on days 3, 7, and 21 following
183 surgery. Eight rats per model were used for naive, sham, and
184 SNT day 3, day 7, and day 21.

185 The second group (group 2) were Wistar male rats initially
186 weighing approximately 200–250 g. They underwent SNT
187 surgery as described above. Animals were culled on days 7 and
188 14 following surgery, just after behavior study, and brains were
189 immediately dissected and stored at -80°C . Four rats per
190 model were used for naive and SNT day 7 and day 14.

191 Dissection and Location of the Central Amygdala

192 The central amygdala (CeA) dissection was performed as
193 follows: The brain was placed on a black rubber stopper under
194 a stereomicroscope and maintained at 4°C , dorsal side up. A
195 coronal section was made at approximately bregma -1 and a
196 second cut at approximately bregma -2.75 according Paxinos
197 and Watson atlas with a razor blade. The thickness of the
198 excised tissue was approximately $2400\ \mu\text{m}$. The tissue was
199 placed on a cold stage caudal side up (nose down), and the
200 central amygdala was micropunched with a sample corer (1
201 mm ID $\times$ 3 mm OD) (Fine Science Tools). The total volume
202 of the CeA is approximately $1.6\ \text{mm}^3$, and the wet weight of
203 the punched samples (assuming that the density of the brain is
204 $1\ \text{mg}/\mu\text{L}$) would be around $1.5\ \text{mg}$ with a protein mass
205 between 80 and $200\ \mu\text{g}$. The CeA tissues were stored at -80
206 $^{\circ}\text{C}$.

207 To locate the area of the central amygdala (CeA), brains
208 from sham model rats were sectioned at $10\ \mu\text{m}$ on a cryostat
209 and stained with Nissl blue (Figure S1). Briefly, sections were
210 defatted by immersion in a mixture of chloroform and
211 methanol (1:1, vol/vol) for 1 h and then washed in 100%
212 ethanol, followed by 90% ethanol in water and 70% ethanol in
213 water, after a short wash with water. The sections were
214 immersed 0.1% cresyl violet acetate solution (Sigma) for 3
215 min, washed with water, and dehydrated in 70% ethanol in
216 water, 90% ethanol in water, and 100% ethanol and mounted
217 on a cover slip. Supporting Information, Figure S1, shows Nissl
218 staining of the right-side of the rat brain slice, stereotaxic
219 location at -1.8 relative to bregma.

220 Sample Preparation for 2D-PAGE Analysis

221 The CeA tissue was placed into 1.5 mL siliconized Eppendorf
222 tubes containing $200\ \mu\text{L}$ of hot (60°C) SDS sample buffer
223 (2% w/v SDS in ultrapure water) and sonicated using a Sonic
224 Vibra Cell at amplitude 20% for 6 s four times at 4°C . Two
225 percent SDS solution is a good lysis buffer for brain tissues and
226 readily penetrates cell membranes. After tissue lysis, the
227 samples were heated to 60°C for 1 h and centrifuged at 14000
228 $\times g$ for 15 min at 4°C . The protein concentration of the
229 resulting supernatants was measured using a BCA assay kit
230 (Novagen) with BSA as the standard. Fifty micrograms of
231 protein was loaded into a $500\ \mu\text{L}$ Viva-spin concentrator
232 column (GE Healthcare), diluted with the ultrapure water to a
233 0.5% SDS concentration, and centrifuged at $13000 \times g$ for less
234 than 1 min to a final volume of $100\ \mu\text{L}$. Three hundred
235 microliters of an exchange buffer (8 M urea, 2 M thiourea, and
236 2% CHAPS in water) was added to the sample. Note that the
237 urea solution was deionized with an ion exchanger prior to
238 adding the other chemicals because urea in aqueous solution
239 exists in equilibrium with ammonium cyanate, which can react

with the NH_3^+ of protein side chains (e.g., lysine) and
introduce charge artifacts, leading to additional spots on the
isoelectric focusing (IEF) gel. The sample was centrifuged at
 $13000 \times g$ for 5 min to a final volume of $100\ \mu\text{L}$, and this step
was repeated 7 times. The final volume of each sample was
around $100\ \mu\text{L}$. The protein concentration was measured using
the RC DC Protein Assay Kit II with BSA standard (Bio-Rad
Laboratories, UK). Just before isoelectric focusing to each
protein sample was added DTT to a final concentration 20
mM, and the samples were ultracentrifuged at $40000 \times g$ for 20
min.

First Dimension of Gel Electrophoresis

Immobiline Dry Strip gels with a nonlinear gradient of pH 3–
11 NL were from GE Healthcare. We used 11 and 24 cm
immobilized pH gradient (IPG) strips. The IPG strips were
passively rehydrated overnight at room temperature to their
original thickness of 0.5 mm with a solution containing
DeStreak rehydration buffer (Amersham, UK) and 0.5% IPG
buffer, pH 3–11 (GE Healthcare), covered by a layer of
silicone oil to prevent them from drying out. We found that
DeStreak rehydration solution was more suitable for this
protocol, as it prevented nonspecific oxidation and streaking
and also significantly improved reproducibility. After rehydra-
tion, the strips were transported to the Protean IEF focusing
tray (Bio Rad). It is important to place electrode wicks in
ultrapure water after soaking on each IPG strip at the cathode
and anode ends. The electrode wicks served as receptacles for
salt in the sample and improved the quality of the IEF step.
sample loading wells were placed at the anodic side of the IPG
strips and were covered with mineral oil. Sample volumes of
40– $100\ \mu\text{L}$ were transferred by pipet into the sample loading
wells. Protein sample load was $60\ \mu\text{g}$ per IPG strip. Isoelectric
focusing was performed using Protean IEF cell system using
the following program: 20°C , 50 μA per strip; step 1, step-n-
hold, 0.01 h, 50 V; step 2, step-n-hold, 4 h, 50 V; step 3, step-n-
hold, 0.01 h, 500 V; step 4, step-n-hold, 4 h, 500 V; step 5,
gradient, 4 h, for 11 cm pH 3–11 NL IPG strips 6000 V or for
24 cm pH 3–11 NL IPG strips 10000 V; step 6, step-n-hold,
99 h, for 11 cm pH 3–11 NL IPG strips 6000 V or for 24 cm
pH 3–11 NL IPG strips 10000 V. The IEF for 11 cm IPG
strips to a total focusing time of 60000 V·h and that for 24 cm
IPG strips was performed up to 100000 V·h.

Second Dimension of Gel Electrophoresis

Upon completion of IEF, the IPG strips were equilibrated to
allow the proteins separated according to their pI values to
fully interact with SDS. The IPG strips were incubated for 10–
15 min in the equilibration buffer (50 mM Tris-HCl (pH 8.8),
containing 2% (w/v) SDS, 1% (w/v) dithiothreitol (DTT), 6
M urea, and 30% (w/v) glycerol). This is followed by a further
10–15 min equilibration in the same solution containing 4%
(w/v) iodoacetamide instead of DTT. The latter step was used
to alkylate any free DTT, as otherwise it migrates through the
second-dimension SDS-PAGE gel, resulting in an artifact
known as point-streaking that can be observed after silver
staining. More importantly, iodoacetamide alkylates sulfhydryl
groups and prevents their reoxidation. After equilibration, the
IPG strips were washed in a gel electrophoresis running buffer
then placed onto the surface of the SDS-PAGE gels and
covered with a layer of a 0.5% agarose in the running buffer
with a pinch of bromophenol blue dye. For 24 cm IPG strips,
the second dimension (SDS-PAGE) was performed in vertical
12% polyacrylamide gels using an Ettan DALTsix System

(Amersham) with the running buffer in the upper chamber and in the lower chamber, run at 20 °C, 200 V until the bromophenol blue dye reached 3/4 of the gel boarder. Four vertical SDS-PAGE gels (260 × 200 × 1.00 mm³) were prepared from a mixture of 75 mL of 4× ProtoGel Resolving buffer (1.5 M Tris-HCl, 0.4% SDS, pH 8.6, National Diagnostics), 90 mL of acrylamide-bis ready-to-use solution 40% (Merck RGaA), 135 mL of HPLC grade water, 300 μL of TEMED, and 3 mL of 10% APS solution. The mixture was filtered through a 0.2 μm filter (Sartorius Stedim Biotech, UK) and then poured into the caster gel DALTsix (VWR) loaded with four glass plate DALT (260 × 200 × 1.00 mm³). For 11 cm IPG strips, second dimension (SDS-PAGE) was carried out simultaneously on 4 slab polyacrylamide gels in a Bio Rad Protean II xi Cell system (Bio Rad) and run at 20 °C at 50 V until the bromophenol blue dye migrated to the end of the stacking gels followed by 200 V until the dye migrated to the bottom of the gels. Four SDS-PAGE gels (125 × 65 × 1.0 mm³) were prepared for resolving gel from a mixture of 36 mL of acrylamide-bis ready-to-use solution 40% (Merck RGaA), 30 mL of 4× ProtoGel Resolving buffer (1.5 M Tris-HCl, 0.4% SDS, pH 8.6, National Diagnostics, UK), 54 mL of HPLC grade water, 72 μL of TEMED, and 720 μL of 10% APS solution. A stacking gel was prepared as follows: 8 mL of acrylamide-bis ready-to-use solution 40% (Merck RGaA), 8 mL of Stacking buffer (PAGE buffer solution pH 6.8, BDH, VWR), 30 mL of glycerol, 34 mL of HPLC grade water, 24 μL of TEMED, and 240 μL of 10% APS solution. All solutions were filtered before casting the gels as previously described. Gels were fixed in a fixing solution (45% ethanol, 45% water, 10% acetic acid) overnight and then stained using a silver staining mass spectrometry compatible kit (Thermo Fisher Scientific, UK) according to the manufacturer's instructions.

Image Acquisition and Spot Detection

Three biological replicate gels from each model, naive, sham, and SNT day 3, day 7, day 14, and day 21, were imaged using a Fujifilm Imager Reader LAS3000 immediately after the second dimension SDS-PAGE. The exposure time was optimized to achieve maximum dynamic range. Images were exported to TIFF format and loaded for spot detection using Progenesis SameSpots V 3.0 (Nonlinear Dynamics, UK). Gel images were aligned by automated calculation of alignment vectors after assigning manually 30 landmark vectors. Automatic analysis was performed on all aligned images using the analysis wizard. All the settings were left as defaults throughout the analysis. For protein identification by MS analysis, a significance level of $p < 0.1$ and spot area ≤ 50 were used to select spots to be excised from the gel. The protein abundance of each spot in the images corresponding to the SNT and control samples was determined as a ratio compared with the intensity of the corresponding spot present in the known control image run in the same physical gel. Normalized volumes of the matched spots across all the gels were further used to evaluate the statistical significance of differences between the SNT and control samples by Student's two-tailed t test.

In-Gel Digestion of Spots from 2D Gels

The stained protein spots with fold change higher than 2 were excised from the gel; a control piece of gel also was cut from a blank region and processed in parallel with other spots. The gel pieces were destained according to the manufacture's protocol (Thermo Fisher Scientific, UK) and washed with 200 μL of ultrapure water five times, followed by dehydration with 200

μL of 100% acetonitrile for 10 min. Organic solvent was removed in a SpeedVac concentrator. Proteins were reduced with 10 mM dithiothreitol for 30 min at 37 °C, followed by alkylation with 55 mM iodoacetamide (IAA) for 60 min in the dark, and then washed with 25 mM ammonium bicarbonate solution twice for 10 min. The gel pieces were dehydrated twice with 80% acetonitrile in 0.3% formic acid and dried in the SpeedVac. To the dried gel pieces was added 20 μL of 12.5 ng/μL trypsin (Promega, UK) in 25 mM ammonium bicarbonate, and the reactions were incubated overnight at 37 °C. The digestion was stopped by addition of formic acid to final concentration of 0.3%. For the MALDI-TOF/TOF-MS analysis, the supernatant was removed into a clean new Eppendorf vial, and the gel pieces were incubated with 50 μL of 50% acetonitrile in 0.1% formic acid for 10 min, following by sonication for 20 min. The supernatants containing peptides were again collected into the same vial, and the gel pieces were dehydrated with 50 μL of 100% acetonitrile for 10 min, followed by sonication for 20 min. The supernatants were pooled, and organic solvent was removed in the SpeedVac to the final sample volume of around 10 μL. The peptide mixture was desalted on reversed phase C₁₈ tips (Millipore) according to the manufacturer protocol, and the bound peptides were eluted in 1.5 μL of matrix (10 mg of α -cyano-4-hydroxycinnamic acid in 70% acetonitrile in 0.5% formic acid) and directly spotted onto the MALDI plate. For the nano-LC-MS/MS analysis, peptides were desalted on in-house manufactured reverse-phase tips, consisting of a plug of C₁₈ matrix (3 m, Empore) packed into a 10 μL tip. Samples were acidified to 1% trifluoroacetic acid, and tips were prewetted and acidified with 100% methanol and 0.5% acetic acid, prior to application. Tips were washed with 0.5% acetic acid, and peptides were eluted with 0.1% trifluoroacetic acid in 75% acetonitrile and lyophilized in a vacuum concentrator. Lyophilized peptides were resuspended in 3 μL of 0.5% acetonitrile in 0.1% TFA and analyzed by nano-LC-ES-MS/MS.

MALDI-TOF/TOF-MS Analysis of 2D-PAGE Excised Spots

MS/MS measurement was carried out on a 4700 Proteomics-Analyzer (Applied Biosystems). The spectra were recorded in reflector mode in a mass range from 700 to 4000 Da with a focus mass of 2000 Da. If the autolytic fragment trypsin with the monoisotopic $[M + H]^+$ m/z at 842.510 or 2211.104 reached S/N of at least 10, an internal calibration was automatically performed using this peak for one-point-calibration. The internal calibration was automatically performed as one-point-calibration if the monoisotopic arginine $[M + H]^+$ m/z at 175.119 or lysine $[M + H]^+$ m/z at 147.107 reached S/N of at least 5. The MALDI-MS resolution for the peptides was ~ 10000 , and the mass accuracy was 10–20 ppm. The MS/MS resolution was 3000–6000. After calibration, a combined database search of MS and MS/MS measurements was performed using the GPS Explorer software (Applied Biosystems, UK) with the following settings: (a) MS peak filtering, mass range from 700 to 4000 Da, minimum S/N filter of 10, mass exclusion list contained background peaks and trypsin fragments with an exclusion tolerance of 100 ppm; (b) MS/MS peak filtering, mass range from 60 Da to a mass that was 20 Da lower than the precursor mass; (c) database search, precursor tolerance 35 ppm and MS/MS fragment tolerance 0.3 Da. Peak lists were compared with the Swiss-Prot or NCBI database restricted to *Rattus*

taxonomy using the MASCOT search engine (Matrix Science). Peptide mixtures that yielded at least twice a MOWSE score² of at least 50 for Swiss-Prot and 63 for NCBI results, respectively, were defined as positive identifications.

Identification of High Molecular Weight Membrane Proteins Using 1D-SDS-PAGE Gel

One-dimensional SDS-PAGE was performed using standard methods on the Hoefer Mighty Small gel system (Amersham). Membrane protein samples were prepared as described below, but the final membrane pellet was reconstituted in 2% SDS, 50 mM Tris-HCl, pH 6.8. Membrane protein samples were heated at 50 °C for 10 min in a solubilization buffer (2% SDS, 20% glycerol, 0.01% bromophenol blue, and 50 mM Tris-HCl, pH 6.8) and 0.1 M dithiothreitol. Membrane protein (5 µg) was resolved on a self-made 10% acrylamide gel. Gels were placed in a fixing solution (45% ethanol, 45% water, 10% acetic acid) overnight and then stained using a silver staining mass spectrometry compatible kit (Thermo Fisher Scientific, UK) according to the manufacturer's instructions.

In-Gel Tryptic Digestion and Peptide Extraction

The molecular weight region on the SDS-PAGE gel between 230 and 80 kDa was divided into five approximately equal bands, and each band was excised with a scalpel. Bands were washed in distilled water and destained according to the manufacturer's protocol. Bands were cut into fine pieces to increase the surface area and dried with acetonitrile and then in a SpeedVac (Thermo Savant) for 5 min. Digestion was carried out using sequencing-grade modified trypsin (approximately 75 ng/µL) (Promega, UK) in 25 mM NH₄HCO₃. Sufficient trypsin solution was added to swell the gel pieces, which were kept on ice for 30 min and then covered with 25 mM NH₄HCO₃ and incubated at 37 °C overnight. Peptides were extracted from the gel pieces with ultrasonication, using sequential washings with a solution of 5% formic acid (FA) in 50% acetonitrile and 70% methanol in 0.1% FA. The extracts were combined, dried in a SpeedVac, and reconstituted in 5 µL of 0.2% FA; following C18 desalting (Empore Octadecyl C18, Sigma), the samples were analyzed by nano-LC-MS/MS.

Nano-LC-ES-MS/MS Analysis of the Excised Spots from 2D-PAGE and Tryptic Digested Peptides from 1D-SDS-PAGE Gel Excised between Gel Regions 80 kDa to 230 kDa

The LC-MS/MS system consisted of an LTQ Orbitrap XL or an LTQ Orbitrap Velos (Thermo Fisher Scientific) equipped with an ESI probe coupled to a Dionex Ultimate 3000 HPLC system (Dionex, UK) fitted with a nanospray ion source (Proxeon, UK). The samples were injected through a 10 µL loop in a pick-up injection mode, 1 µL per sample. The injected peptides were separated on a C18 analytical column, which was prepared by packing a Picotip spray emitter (150 mm length, 100 µm internal diameter, New Objective) with ProntoSIL C18 phase matrix (120 Å pore size, 3 µm bead size, Bischoff Chromatography). Peptides were separated using two different gradient programs with a mobile phase A of 0.1% formic acid in water and mobile phase B of 0.1% formic acid in 80% acetonitrile. The first one provides a separation of the majority of peptides, requires a shorter time, and was used for peptides extracted from spots of 2D gels. The second longer gradient was developed in order to provide more separation of peptides and increase the chance to detect low abundance peptides and more hydrophobic peptides derived from the 1D-gel band of membrane proteins. The shorter LC gradient was

as follows: After 3 min with 2% B, the mobile phase composition was raised to 5% B and was raised again to 20% over 12 min, and then to 50% B over the next 15 min, and then it was raised sharply over 0.2 min to 90% B and maintained at 90% B for a further 5 min, before returning to 2% B to recondition the column at 2% B for 5 min. The total gradient time was 40 min. The flow rate was 300 nL/min, and the eluent was directed to the ESI source of the LTQ-Orbitrap mass spectrometer. The second gradient was used for the identification of membrane proteins and was as follows: After 3.1 min at 2% B, the proportion of B was raised to 15% B over the next 32 min using gradient curve 8 (Chromeleon software, Dionex, UK). The proportion of B was then increased to 60% over 75 min, before returning to 20% B in 0.1 min. The column was re-equilibrated for a 10 min giving a total run time of 120 min. The flow rate was 300 nL/min, and the eluent was directed to the ESI source of the LTQ-Orbitrap mass spectrometer. MS data was acquired using a data-dependent acquisition mode, and operated at 60000 resolution (full width at half-maximum height, fwhm definition), and the top five 2+, 3+, and 4+ ions in the 300–1800 *m/z* range were selected for MS/MS. Charge state 1+ ions were rejected. The automatic gain control for the Orbitrap was set to 500000 ions, and the automatic gain control for the MS/MS in the ion trap was set at 10000 ions. For MS/MS, the isolation width was set at 2; the collision energy was 35%. Three MS/MS microscans for each precursor were accumulated. Maximum injection time into the ion trap in MS/MS was 200 ms, and maximum accumulation time in the Orbitrap was 500 ms. Dynamic exclusion was enabled, and selected ions were excluded for 180 s before they could be selected for another round of MS/MS.

Protein Identification

MS/MS peak lists were converted to mzXML format using ReAdW, version 4.4.1 (LTQ XL Orbitrap data). This tool was used with default parameters except that zlib compression of spectral data was enabled. Data was uploaded to the central proteomics facilities pipeline (CPFP at https://cpfp-master.molbiol.ox.ac.uk/cpfp_demo/auth/login) or <https://mascot.molbiol.ox.ac.uk> (www.proteomics.ox.ac.uk). Files were searched using Mascot, version 2.3.01 (Matrix science), X! TANDEM, version 2008.12.01.1, and OMSSA, version 2.1.8, against a concatenated target and reversed decoy version of the IPI rat sequence database (EBI), version 3.79 containing 78946 target protein sequences and 41 806 600 residues. Enzyme was set to trypsin (or chymotrypsin for some experiments) allowing for up to 3 missed cleavages. Carbamidomethyl (C) was set as a fixed modification, and acetyl (N-term), deamidated (NQ), and oxidation (M) were set as variable modifications. Mass tolerances for MS and MS/MS peak identifications were 20 ppm and 0.6 Da, respectively. The level of confidence for peptide identifications was based on the Mascot assignment of "identity" (*p* < 0.05). Protein identifications were only made when two or more peptides from that protein were identified with Mascot scores above the identity threshold. In cases where the identified protein was a member of a multiprotein family with similar sequences, the protein identified was the one with the highest number of matched peptide identification rate.

Classification of Differentially Expressed Proteins Identified by 2D-PAGE MS

Database searches were carried out using BLAST algorithm at the National Center for Biotechnology Information (NCBI),

550 and the ProteinCenter, version 3.3 (<http://thermo.proteincenter.proxeon.com/ProXweb/app>), and KEGG data-
551 base (www.genome.jp/kegg) were used to obtain general
552 information concerning the biological function of the differ-
553 entially expressed proteins from gel- and MS-based proteomics.
554 The Gene Ontology (GO) annotations of proteins were
555 compared with the ones from a reference proteome (e.g.,
556 identified proteins vs the entire protein database or a subset of
557 the identified proteins vs the overall identified proteins).

559 Membrane Protein Purification by Differential 560 Centrifugation

561 CeA tissues were placed into an ice-cold glass homogenizer,
562 along with approximately 1 mL of homogenization buffer for
563 every 0.1 g of tissue, and sonicated using the Sonic Vibra Cell
564 at amplitude 20% for 6 s four times at 4 °C. The
565 homogenization buffer contained 0.25 M sucrose, 50 mM
566 TEAB, pH 7.4, at 4 °C, and protease inhibitor cocktail (Sigma-
567 Aldrich, USA). All solutions were prepared in ultrapure water
568 (Fluka, UK). After sonication, the samples were left on ice for
569 1 h. Membrane fractions were prepared using differential
570 centrifugation as follows: an initial centrifugation at 10000 × *g*
571 for 10 min at 4 °C was used to sediment the cell debris, nuclei,
572 and unbroken cells. The supernatant was centrifuged at
573 100000 × *g* for 30 min at 4 °C. The pellets from this step
574 were resuspended in 100 mM sodium carbonate (Sigma-
575 Aldrich, USA) and left for 30 min on ice. Samples were then
576 centrifuged at 100000 × *g* for 30 min at 4 °C. The resultant
577 pellets were washed three times with ultrapure water and then
578 resuspended in 40 μL of 0.2% Protease MAX Surfactant
579 (Promega, UK) in 50 mM TEAB. The samples were mixed by
580 vortexing for 2 min at medium speed. Proteins were found to
581 become soluble within 40 min. All samples were stored at −80
582 °C. The membrane protein concentrations were determined
583 using the BCA assay kit.

584 In-Solution Digestion of Membrane Proteins

585 Five micrograms of protein of each sample was taken for an
586 on-column stable isotope dimethyl labeling and put into new
587 Eppendorf tubes. The samples volume was adjusted to 93.5 μL
588 with 50 mM TEAB, and 1 μL of 0.5 M DTT was added. The
589 samples were then incubated at 56 °C for 20 min, followed by
590 addition of 2.7 μL of 0.55 M iodoacetamide and incubation at
591 room temperature in the dark for 15 min. After this, 1 μL of
592 1% Protease MAX Surfactant and 1.8 μL of 1 μg/μL Trypsin
593 Gold (Promega) were added to the samples, which were left
594 overnight at 37 °C in a water bath. The samples were
595 centrifuged at 12000 × *g* for 10 s, and digestion was stopped by
596 addition of 5 μL of formic acid (Merck). Note that Protease
597 MAX Surfactant is a hydrophobic anionic surfactant (sodium-
598 3((1-(furan-2-yl)undecyloxy)carbonylamino)propane-1-sulfo-
599 nate) and degrades over the course of a digestion reaction.
600 However, for a complete degradation of the surfactant, the
601 samples were incubated for 10 min at 37 °C. The
602 concentration of peptides was determined by UV-spectrometry
603 using an extinction coefficient of 1.1 for 0.1% (g/L) solution at
604 280 nm.

605 On-Column Triplex Stable Isotope Dimethyl Labeling of 606 the Membrane Proteins

607 Stable isotope dimethyl triplex labeling at the peptide level was
608 performed as described by Boersema et al.¹⁷ Equal amounts of
609 protein were labeled on-column using “light”, “intermediate”,
610 and “heavy” dimethyl labeling reagents, and the three channels

were mixed in a 1:1:1 ratio. In brief, 5 μg of tryptic digested
611 proteins per channel in 50 mM TEAB, 5% formic acid were
612 applied on individual homemade μC18 Stage Tips. The μC18
613 Stage Tips were made as described by Neugebauer et al.³
614 Briefly, 10 μL tips were blocked with 3 mg of Sep-Pak Classic
615 C18 stationary phase (Waters) sandwiched with another layer,
616 and the adapter with a 1 mL syringe was used as a pressure
617 device. The μC18 tips were prewashed three times with 450
618 μL of acetonitrile, followed by three washes with 0.6% (v/v)
619 acetic acid in water. This was followed by loading each of the
620 three samples on a separate μC18 tip. The μC18 Stage tips
621 were washed with 450 μL of 0.6% (v/v) acetic acid in water
622 followed by application of 225 μL of the respective stable
623 isotope dimethyl labeling solution (“light”, “intermediate”, or
624 “heavy”). The on-column labeling solutions were prepared per
625 sample or label. Four hundred fifty microliters of 50 mM
626 sodium phosphate buffer, pH 7.5, was mixed with 25 μL of 4%
627 (v/v) formaldehyde in ultrapure water (CH₂O (37% (v/v), cat
628 no. 25,254-9, Sigma), CD₂O (20%, 98% D, Isotec), or ¹³CD₂O
629 (20%, 99% ¹³C, 96% D, Sigma-Aldrich, USA) and 25 μL of 0.6
630 M cyanoborohydride in ultrapure water (sodium cyanobor-
631 ohydride or sodium cyanoborodeuteride, 96% D). The
632 resulting eluates were reappplied ten times on the same μC18
633 Stage tips to allow complete labeling, and this took at least 30
634 min. After this, the μC18 Stage tips were washed with 450 μL
635 of 0.6% (v/v) acetic acid in water. The labeled peptides were
636 eluted from the μC18 Stage tips with 150 μL of 0.6% (v/v)
637 acetic acid in 80% acetonitrile. The differently labeled samples
638 were mixed and dried in a SpeedVac to 10 μL, then acidified to
639 0.25% with formic acid, and immediately analyzed by nano-
640 LC-MS/MS.

642 Nano-LC-MS/MS Analysis of Triplex Stable Isotope 643 Dimethyl Labeled Peptides and Data Analysis

The LC-MS/MS system consisted of a Q Exactive mass
644 spectrometer (Thermo Fisher Scientific, UK) equipped with a
645 nano-ESI probe coupled to an Ultimate 3000 RSLCnano
646 system (Dionex, UK). Chromatographic separation was
647 performed using a NanoEASY (Thermo Fisher Scientific)
648 C₁₈ reverse-phase column (50 cm long, 75 μm internal
649 diameter, 2 μm beads) at a flow rate of 300 nL min^{−1}. Mobile
650 phase A consisted of 0.1% formic acid in 5% acetonitrile.
651 Mobile phase B consisted of 0.1% formic acid in 80%
652 acetonitrile. A 290 min gradient was used to separate the
653 dimethyl labeled peptides, and each sample was typically
654 injected three times and data were merged in order to increase
655 sample coverage. The LC gradient method was as follows:
656 After 20 min at 5% B, the proportion of B was raised to 30% B
657 over the next 180 min (using gradient curve 8 Chromeleon
658 software, Dionex, UK). The proportion of B was then
659 increased to 50% over 60 min and further increased to 98%
660 over the next 10 min before returning to 5% B in 0.1 min. The
661 column was re-equilibrated for 10 min. The eluent was
662 directed to the ESI source of the Q Exactive mass
663 spectrometer. The mass spectrometer was operated in a “top
664 10” data-dependent acquisition mode with dynamic exclusion
665 enabled (40 s). Survey scans (mass range 300–1650 Th) were
666 acquired at a resolution of 70000 at 200 Th with the ten most
667 abundant multiply charged (*z* ≥ 2) ions selected with a 3-Th
668 isolation window for fragmentation by higher-energy collisional
669 dissociation. MS/MS scans were acquired at a resolution
670 of 17500 at 200 Th.

672 Protein Identification and Quantification

673 Quantification of peptide triplets was processed using
674 Proteome Discoverer 1.3, version 1.3.0.339 (Thermo Fisher
675 Scientific). MS/MS peak lists were converted to mgf format
676 using ProteoWizard msconvert, release 3.0.3535 (Q Exactive
677 data). Searches against the UniProt rat database^{20,21} were
678 performed using Mascot (version 2.3.01). Mascot was set up in
679 its special quantification mode in which N-terminal and lysine
680 demethylation was set as an interconnected modification (i.e.,
681 when the peptide is dimethyl labeled on a lysine residue, the N
682 terminus is also expected to be labeled with the same
683 isotopomeric dimethyl label and vice versa) to lower the
684 number of false-positive identifications of peptides with
685 different isotope labels. We treated three technical nano-LC-
686 MS/MS runs as replicates and combined them during software
687 analysis generating one .msf file corresponding to one
688 biological replicate. Two biological replicates were used.
689 Trypsin was chosen as cleavage specificity allowing two missed
690 trypsin cleavage sites and maximum modifications per peptide
691 of 4. Search parameters were precursor mass tolerance 10 ppm
692 and fragment mass tolerance 0.1 Da. Carbamidomethylation
693 (C) was set as a static modification. Dynamic modifications
694 were oxidation (M), dimethyl (K), dimethyl (N-term),
695 dimethyl-4 (K), dimethyl-4 (N-term), dimethyl-8 (K), and
696 dimethyl-8 (N-term). The peptide validator node was set to
697 "false". The "RT tolerance of isotope pattern multiplets"
698 parameter was set to 0.3 min. We required proteins to have a
699 minimum of two quantifiable unique peptides. Proteome
700 Discoverer Peptide results were filtered to 1% false discovery
701 rate, peptide length of 6–35, peptide score >20%, maximum
702 search engine rank 1, and peptide mass deviation of 7 ppm. For
703 reporting the quantitation data, we set fold change higher than
704 2 and FDR $\geq$ 0.5%. Proteins that were not reproducibly
705 quantified across biological replicates were removed from
706 downstream analysis. Then, all the quantification results were
707 normalized against the log₂ of the median ratio of all the
708 quantified peptides and reported as the log₂ day 3 (or day 7)
709 or day 21 to sham.

710 The mass spectrometry proteomics data have been
711 deposited to the ProteomeXchange²² Consortium via the
712 PRIDE²³ partner repository with the data set identifier
713 PXD017473

714 Western Blot

715 Fifteen micrograms of protein from CeA tissue lysates (these
716 samples were prepared as described in [sample Preparation for](#)
717 [2D-PAGE Analysis](#)) and the membrane protein (samples were
718 prepared as described in [Membrane Protein Purification by](#)
719 [Differential Centrifugation](#)) samples (sham, $n = 3$; naive, $n = 3$;
720 SNT days 3, 7, and 21, $n = 3$) in 100 mM Tris-HCl, pH 6.8,
721 30% glycerol, 2% SDS, 0.0025% bromophenol blue, and 100
722 mM DTT were heated at 60 °C for 20 min and then were
723 loaded on 10% polyacrylamide/bis-acrylamide TRIS-HCl
724 discontinuous gels. Proteins were transferred onto poly-
725 (vinylidene fluoride) (PVDF) membranes (GE Healthcare)
726 using the iBlot DRY Blotting system for 15 min at 20 V
727 (Invitrogen, UK). Membranes were washed three times in
728 TBS, 0.1% Triton X-100 (TBS-T) for 10 min each time and
729 then blocked in 5% BSA, TBS-T for 1 h at RT and
730 subsequently washed three times for 5 min in TBS-T.
731 Afterward, the membranes were incubated with anti-DCX
732 (1:700, ab23542, Abcam) in 5% BSA, TBS-T for 1 h at RT.
733 Following three washes, the membranes were incubated with

the secondary goat anti-rabbit HRP antibody (1:1000, Dako) 734
in 5% BSA, TBS-T for 1 h at RT. The PVDF membranes were 735
then washed three times with TBS-T, and bands were 736
visualized using a chemiluminescence kit (AppliChem, UK) 737
and detection with the Fujifilm LAS-3000 Imaging System. 738
Ponceau staining was used as control for equal protein loading 739
and transfer. 740

Immunohistochemistry 741

To study the colocalization of doublecortin (DCX) with cell 742
compartment markers, a triplex-labeling immunohistochemis- 743
try technique was used. We used three representative animals 744
each from SNT day 7 and day 21 rats. After embedding the 745
dissected brain tissue as described above in OCT compound 746
(Sakura Finetek), 10 μ m thick coronal sections were cut on a 747
cryostat, mounted on poly(L-lysine)-coated glass slides, and 748
air-dried for 3 h at room temperature. The first and third 749
sections were mounted on positively coated glass slides, three 750
sections per slide. Five slides per animal were selected per 751
brain region for each immunostaining. The brain sections were 752
rinsed three times for 10 min in PBS and then postfixed in 4% 753
paraformaldehyde in phosphate buffer for 15 min, followed by 754
again washing three times in phosphate buffer. Afterward, 755
sections were incubated in blocking solution (PBS, pH 7.4, 756
0.3% Triton X-100, 2 mg/mL BSA, 5% normal goat serum for 757
60 min at RT. Primary antibodies were diluted in fresh 758
blocking solution and applied overnight at 4 °C. The primary 759
antibodies used were as follows: anti- α 1 Na,K-ATPase (1:200, 760
mouse monoclonal; ab7671), anti-58K Golgi protein (1:200, 761
mouse monoclonal; ab27043), anti-doublecortin (1:100, rabbit 762
polyclonal; ab23542) from Abcam. Next, the brain sections 763
were washed in PBS for 30 min at room temperature and 764
subsequently incubated for 2 h in the dark with the appropriate 765
fluorescent secondary antibody (anti-mouse or anti-rabbit IgG 766
conjugated with FITC-Red or TRITC-Green, Dako, UK) 767
diluted 1:200 in blocking solution. Following three additional 768
rinses in PBS, sections were finally mounted with cover slips 769
using Vectashield water-based mounting medium with DAPI 770
(Vector Laboratories). The analysis of negative controls 771
(omission of primary antibody) was simultaneously performed 772
in order to exclude the presence of nonspecific immuno- 773
fluorescent staining, cross-immunostaining, or fluorescence 774
bleed-through. Samples were analyzed on a wide-field Nikon 775
80i microscope, and pictures were acquired with an ultrahigh- 776
quality Nikon DXM1200F digital camera controlled with 777
LUCIA G software. 778

Quantitative Real-Time PCR Analysis 779

For quantitative RT-PCR, RNA from sham and SNT day 3, 780
day 7, day 14, and day 21 CeA tissues was extracted using a 781
peqGOLD MicroSpin Total RNA kit (Qiagen, UK). One 782
nanogram of each RNA sample was used per analysis. The 783
RNA was then reverse transcribed using the SuperScript III 784
First-Strand Synthesis System kit (Invitrogen). Real-time PCR 785
was performed using the StepOnePlus System and the 2x Fast 786
SYBR Green Master Mix (Applied Biosystems) to accurately 787
determine target gene expression levels. PCR reactions were 788
performed in a total reaction volume of 20 μ L, containing 10 789
 μ L of SYBR Master Mix, 8 μ L of 0.125 ng/ μ L cDNA for each 790
model, and 2 μ L of both forward and reverse primers (10 μ M 791
each). Samples were loaded in triplicate on the plate for each 792
primer set, and both GAPDH and yWHAZ were used as 793
housekeeping genes. The PCR was run with an initial step of 794
95 °C for 20 s to activate the AmpliTaq Fast DNA polymerase 795

Figure 1. Silver stained 2D gel of (a) the right-side CeA and (b) the left-side CeA. Thirty-three spots were differentially detected with $F \geq 2$ as shown by arrows. Progenesis SameSpot software was utilized for the cross-comparison analysis between 2D gels of naive rats and rats at days 7 and 14 after SNT surgery. The first dimension, an isoelectric focusing (IEF), was performed with an immobilized pH gel (pH 3–11 NL) 11 cm long, where proteins separated according to pI values. The second dimension, SDS-PAGE, was performed with a 12% SDS polyacrylamide gel, where the proteins are separated by their molecular weight.

enzyme, followed by 40 cycles of 95 °C for 3 s, 59 °C (annealing) for 15 s, and 72 °C for 15 s. Fluorescence

incorporation of the SYBR Green to double stranded DNA was measured after each cycle to generate an amplification curve.

Table 1. Proteins Identified by Nano-LC-MS/MS of Spots Excised from 2D Gels^a

spots	accession no. (SwissProt)	protein description	mascot score	MW	pI	seq cov (%)	fold change comparison between right- and left-sides of CeA in naive and SNT day 7 and day 14 rats, 2 < F > 3
297	P15999	ATP synthase subunit α , mitochondrial	865	59831	9.22	38.2	right-side of SNT day 7
297	Q5XI77	Anxa11 RCG39189, isoform CRA_a	821	54468	7.53	38.6	
297	Q6P6V0	glucose-6-phosphate isomerase	457	62958	7.38	22.2	
429	P16617	phosphoglycerate kinase 1	702	44909	8.02	53.0	right-side of SNT day 7
429	Q8VHF5	citrate synthase, mitochondrial	494	52176	8.53	29.4	
429	P05065	fructose-bisphosphate aldolase A	173	39783	8.31	25.3	
429	P15999	ATP synthase subunit α , mitochondrial	124	59831	9.22	23.0	
429	Q9JJ54	heterogeneous nuclear ribonucleoprotein D0	94	38339	7.62	17.0	
429	P17764	acetyl-CoA acetyltransferase, mitochondrial	92	45009	8.92	14.9	
783	Q6P7Q4	lactoylglutathione lyase	224	20977	5.12	36.4	right-side of SNT day 7
783	P28073	proteasome subunit β type-6	205	25502	4.85	16.8	
783	O35509	Ras-related protein Rab-11B	160	24588	5.64	27.1	
902	P10111	peptidyl-prolyl cis-trans isomerase A	124	18091	8.34	31.7	right-side of naive
948	P15999	ATP synthase subunit α , mitochondrial	2814	59831	9.22	67.3	right-side of naive and SNT day 14
948	Q6AXS5	plasminogen activator inhibitor 1 RNA-binding protein	94	44841	8.6	19.0	
948	Q8R4T5	general receptor for phosphoinositides 1-associated scaffold protein	39	42637	9.16	17.0	
951	P54313	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β -2	564	38048	5.6	33.5	right-side of naive
951	P42123	L-lactate dehydrogenase B chain	254	36874	5.7	27.5	
951	P81799	N-acetyl-D-glucosamine kinase	213	37458	5.55	11.7	

^a11 cm IPG strip corresponding to the 1st group of neuropathic pain rat models. The central amygdala was excised from the left- and right-sides of rat brain. 2D gels of the left- and right-sides of the CeA were compared using Protein Progenesis Software; protein spots with a fold-change higher than or equal to 2 and $p < 0.1$ were excised, and proteins were extracted and digested with trypsin followed by nano-LC-MS/MS analysis on the Orbitrap Velos Pro mass spectrometer.

Product composition was verified at the end of each reaction with melt curve analysis in 0.6 °C increments from 60 to 95 °C. Primer sequences (5' to 3') with annealing temperatures of 60 °C produce amplicon size <250 bp.

RESULTS

Identification of Differentially Expressed Proteins in Neuropathic Pain Models by 2D Gel Analysis

The objective of this work was to identify the proteins with altered expression levels in the central amygdala of animals with neuropathic pain. Identifying these proteins would be valuable for understanding the mechanism of neuropathic pain. We first studied protein expression profiles in the protein extract from rats after and before SNT surgery using 2D gels. The silver-stained protein spots were quantitatively analyzed, and protein spots with a fold-change higher than or equal to 2 and $p < 0.1$ were considered as a significant change in this work. Protein spots were excised from 2D gels and subjected to in-gel trypsin digestion, and peptides were analyzed by peptide mass fingerprinting using a MALDI-TOF/TOF mass spectrometer (MALDI-TOF/TOF-MS) or alternatively by nano-LC-MS/MS analyses. We performed preliminary studies by capillary LC-ES-MS/MS using the LCQ mass spectrometer to evaluate the protein identification from silver-stained 2D gels prior to more detail studies on the Orbitrap instruments. Nano-LC-MS/MS runs were performed on a nano-LC connected to either of two mass spectrometers, Orbitrap XL

or Orbitrap Velos. Two groups of neuropathic pain rat models were used in this study. For each neuropathic pain model, we generated 2D gels of three biological replicates and three technical replicates. We compared protein changes in the right and left CeA of naive rats versus rats at 7 and 14 days after SNT surgery (group 1), whereas sham or naive rats versus rats at days 3, 7, and 21 after SNT surgery represent group 2. The behavioral results on the animals used in this research were as reported in previous studies.^{24–26} The behavioral data on the animals used in this research have been published previously. We used 11 cm long IPG strips with pH 3–11 for group 1. We detected around 1600 spots per gel (after exclusion of false spots) for both the naive group and SNT group gels. Figure 1 shows a representative 2D gel. We performed gel-to-gel matching of spots with the reference gel, defined to be the gel containing the most spots, and nonmatching spots were removed after careful and rigorous manual validation. We matched around 1500 spots in the CeA gels. Tables 1–5 summarize the differentially expressed proteins. Briefly, we identified the same proteins for these spots by the above two nano-LC-MS/MS systems. It should be taken into consideration that as a maximum of 3000 spots can be observed on a typical 2D gel by silver staining, many proteins will inevitably comigrate to the same spot position, thus confounding their accurate quantitation and mass spectrometric identification, and also some of these proteins maybe expressed in different quantities in the CeA. We found that the products of two genes migrated to the same faint spot on a silver-stained gel.

Table 2. Identified Proteins by Nano-LC-MS/MS from Spots Excised of 2D Gels^a

	spot no.	accession no. (SwissProt)	protein description	mascot score	MW	pI	seq cov (%)	fold change left-CeA	identified by
1	128	Q9ER34	aconitate hydratase, mitochondrial precursor	450	85433	5.80	45	↑ (2.5)	nano-LC-LTQ Orbitrap XL
2	233	Q9QUL6	vesicle-fusing ATPase	356	82652	6.55	20	↑ (2.4)	nano-LC-LTQ Orbitrap XL
3	337	<i>b</i>						↓ (2.5)	nano-LC-LTQ Orbitrap XL
4	459	P07335	creatine kinase B-type	117	42725	5.42	52	↑ (2.9)	nano-LC-LTQ Orbitrap XL
	459	P82995	heat shock protein HSP 90-α	95	84815	5.01	39	↑ (2.9)	nano-LC-LTQ Orbitrap XL
	459	F1LTA7	uncharacterized protein (fragment)	69	42385	5.21	28	↑ (2.9)	nano-LC-LTQ Orbitrap XL
	459	D4A2Z9	uncharacterized protein	54	80700	4.91	31	↑ (2.9)	nano-LC-LTQ Orbitrap XL
	459	F1M5B6	uncharacterized protein (fragment)	42	76900	5.34	22	↑ (2.9)	nano-LC-LTQ Orbitrap XL
	459	D4AE04	uncharacterized protein	39	57300	5.22	25	↑ (2.9)	nano-LC-LTQ Orbitrap XL
	459	D3ZE66	uncharacterized protein	31	64700	5.12	34	↑ (2.9)	nano-LC-LTQ Orbitrap XL
5	472	P11980	pyruvate kinase isozymes M1/M2	234	57819	6.63	21	↓ (2.9)	nano-LC-LTQ Orbitrap XL
6	510	<i>b</i>						↓ (2.6)	nano-LC-LTQ Orbitrap XL
7	519	<i>b</i>						↓ (2.5)	nano-LC-LTQ Orbitrap XL
8	534	Q5XI34	protein phosphatase 2 (formerly 2A)	804	65323	4.75	35	↓ (2.9)	nano-LC-LTQ Orbitrap XL
	534	Q4QQT4	serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A β	789	66006	4.73	23	↓ (2.9)	nano-LC-LTQ Orbitrap XL
	534	Q6P9V9	tubulin α-1B chain	403	50152		25	↓ (2.9)	nano-LC-LTQ Orbitrap XL
	534	P50398	guanosine diphosphate dissociation inhibitor 1, isoform CRA_a	345	50538	4.75	9	↓ (2.9)	nano-LC-LTQ Orbitrap XL
	534	P34058	heat shock protein HSP 90-β	201	83281	5.03	49	↓ (2.9)	nano-LC-LTQ Orbitrap XL
9	548	<i>b</i>						↑ (2.3)	nano-LC-LTQ Orbitrap XL
10	552	O54937	pyruvate dehydrogenase kinase isoform 4	32	46679	6.47	26	↓ (2.7)	nano-LC-LTQ Orbitrap XL
11	578	<i>b</i>							nano-LC-LTQ Orbitrap XL
12	602	P60203	myelin proteolipid protein	70	30077	8.71	19	↓ (2.6)	nano-LC-LTQ Orbitrap XL
13	614	G3V6D3	ATP synthase subunit β	656	56345	5.27	77	↑ (2.7)	nano-LC-Orbitrap Velos Pro MS
	614	P69897	tubulin β-5 chain	220	49671	4.89	54	↑ (2.7)	nano-LC-Orbitrap Velos Pro MS
	614	P06761	78 kDa glucose-regulated protein	131	72347	5.16	58	↑ (2.7)	nano-LC-Orbitrap Velos Pro MS
	614	P07323	γ-enolase	70	47141	5.16	45	↑ (2.7)	nano-LC-Orbitrap Velos Pro MS
14	624	P42123	L-lactate dehydrogenase B chain	253	36612	5.92	37	↓ (2.5)	nano-LC-LTQ Orbitrap XL
15	638	Q497B0	ω-amidase NIT2	241	30701	6.90	15	↓ (3.4)	nano-LC-LTQ Orbitrap XL
16	658	<i>b</i>						↓ (3.6)	nano-LC-LTQ Orbitrap XL
17	674	P48500	triosephosphate isomerase	929	26849	7.29	45	↓ (2.6)	nano-LC-LTQ Orbitrap XL
18	702	Q3T1K5	F-actin-capping protein subunit α-2	512	32967	5.65	30	↓ (1.9)	nano-LC-LTQ Orbitrap XL
	702	P42123	L-lactate dehydrogenase B chain	160	36612	5.92	27	↓ (1.9)	nano-LC-LTQ Orbitrap XL
19	728	O35244	peroxiredoxin-6	501	24819	5.64	29	↓ (1.5)	nano-LC-LTQ Orbitrap XL
20	730	P22062	protein L-isoaspartate (D-aspartate) O-methyltransferase	142	24641	7.14	20	↓ (2.0)	nano-LC-LTQ Orbitrap XL

Table 2. continued

	spot no.	accession no. (SwissProt)	protein description	mascot score	MW	pI	seq cov (%)	fold change left-CeA	identified by
21	740	P09527	Ras-related protein Rab-7a	365	23504	6.40	48	↑ (2.7)	nano-LC-LTQ Orbitrap XL
	740	Q6NYB7	Ras-related protein Rab-1A	292	22678	5.93	41	↑ (2.7)	nano-LC-LTQ Orbitrap XL
	740	P61589	transforming protein RhoA	283	21782	5.83	40	↑ (2.7)	nano-LC-LTQ Orbitrap XL
	740	P10536	Ras-related protein Rab-1B	262	22163	5.55	37	↑ (2.7)	nano-LC-LTQ Orbitrap XL
	740	O35509	Ras-related protein Rab-11B	223	24489	5.64	18	↑ (2.7)	nano-LC-LTQ Orbitrap XL
	740	Q4KM73	UMP-CMP kinase	196	22169	5.66	26	n/d	nano-LC-LTQ Orbitrap XL
22	755	Q5U316	Ras-related protein Rab-35	94	23025	8.52	17	↑(2.5)	nano-LC-LTQ Orbitrap XL
	755	P97576	GrpE protein homologue 1, mitochondrial	43	24297	8.57	15	↑(2.5)	nano-LC-LTQ Orbitrap XL
23	772	P39069	adenylate kinase isoenzyme 1	249	21584	7.66	28	↓ (2.5)	nano-LC-LTQ Orbitrap XL
24	797	P37805	transgelin-3	90	22501	7.44	24	↓ (3.0)	nano-LC-LTQ Orbitrap XL
25	874	Q6LED0	histone H3.1	30	15404	11.71	34	↑(2.5)	nano-LC-LTQ Orbitrap XL
	874	P62804	histone H4	93	11367	11.90	27	↑(2.5)	nano-LC-LTQ Orbitrap XL
	874	P0C0S7	histone H2A.Z	58	13422	11.29	25	↑(2.5)	nano-LC-LTQ Orbitrap XL
26	883	O88767	protein DJ-1	152	19974	6.77	24	↑(2.6)	nano-LC-LTQ Orbitrap XL
	883	P19804	nucleoside diphosphate kinase	200	17283		28	↑(2.6)	nano-LC-LTQ Orbitrap XL
27	899	P63025	vesicle-associated membrane protein 3	100	11480	7.85	31	↓ (2.0)	nano-LC-LTQ Orbitrap XL
	899	Q63228	glia maturation factor β	89	16736	5.32	41	↓ (2.0)	nano-LC-LTQ Orbitrap XL

^a11 cm IPG strip corresponding to the 1st group of neuropathic pain rat models. The central amygdala was excised from the left-side of rat brain.
^bNot determined.

854 However, the identity of protein with the highest MASCOT
855 score was taken, although it may be that low-abundance
856 proteins are a true fold-change. There is lateralization of pain
857 processing in the amygdala.^{18,19,27,28} Higher neuronal activity
858 in the right than in the left CeA after formalin administration
859 and in a model of arthritic pain was previously reported.^{27,29}
860 Therefore, we examined if there are protein changes
861 between the right and the left CeA in naive and SNT model
862 rats (Supporting Information, Figure S2). From these matched
863 spots with the *t* test and 2-fold change as cut off, we found that
864 one spot, 948, was significantly different between the left and
865 right CeA in naive and SNT model rats at day 14, and it was
866 identified as a mitochondrial protein, metabolic enzyme,
867 specifically ATP-synthase subunit α , which produces ATP
868 from ADP in the presence of a proton gradient across the
869 membrane. Four spots were increased with fold change of $2 <$
870 $F > 3$ in the right-side CeA after SNT surgery at day 7 (Table
871 1). Spot 297 was identified as annexin A11, which has complex
872 and essential functions in several biological pathways including
873 apoptosis and proliferation. Spot 429 was identified as
874 phosphoglycerate kinase 1. Phosphoglycerate kinase is a
875 transferase enzyme used in the seventh step of glycolysis;
876 thus deficiency of this kinase is a genetic disorder that affects
877 the body's ability to break down the simple sugar glucose. We
878 found four significantly increased spots in the right-side CeA
879 versus the left-side CeA after SNT surgery at day 14. Spot 902
880 was identified as peptidyl-prolyl cis-trans isomerase A, which

accelerates the folding of proteins. We identified seven proteins
with MASCOT score higher than 50 for spot 951 of the 2D gel
most likely due to post-translational modification and
comigration of similar molecular weight and pI value proteins.
Several guanine nucleotide-binding proteins (G proteins) were
identified with highest MASCOT score for this spot.
We then compared 2D gels of the right CeA from naive
versus SNT rats. Thirty three matched spots showed significant
differences, and 26 of them were identified by nano-LC-MS/
MS and by peptide finger printing using MALDI-TOF/TOF
analyses (Figure 1). These instruments were used to further
verify the identity of proteins, so the proteins that were
identified by two instruments were considered as a true fold-
change. According to Progenesis Same Spot analysis of 2D
gels, 16 spots were down-regulated in the right CeA after SNT
at day 14. Twenty-two protein spots were down-regulated in
the right CeA between days 7 and 14 after SNT surgery. One
protein spot was up-regulated between day 7 and day 14 after
SNT surgery, and two protein spots were up-regulated at day 7
after SNT surgery. A summary of the identified proteins is
presented in Table 2. For example, spot 961 was identified as
calcium/calmodulin-dependent protein kinase type II subunit
 α . This protein is a prominent kinase in the central nervous
system (CNS) that may function in long-term potentiation and
neurotransmitter release. Spot 939 was identified as enoyl-CoA
hydrase, which has neurotrophic and neuroprotective proper-
ties on a broad spectrum of CNS neurons, and it promotes cell

Figure 2. 2D gel of the left-side CeA. Arrows indicate spots with fold change ≥ 2 , when 2D gels of naive rats are compared to 2D gels of neuropathic pain model rats at day 7 and 14 after SNT surgery.

survival. Spot 590 was identified as annexin 3, a protein that exhibits calcium-dependent phospholipid binding. Spot 411 was identified as mitogen-activated protein (MAP) kinases, which are serine/threonine-specific protein kinases that respond to extracellular stimuli and modulate cellular activities, such as proliferation, cell survival, and apoptosis. Spot 728 was identified as peroxiredoxin-6, which belongs to antioxidant enzymes and plays a role in the regulation of phospholipid turnover as well as in protection against oxidative injury. Spot 959 was identified as creatine kinase B. This protein was previously reported as down-regulated after nerve injury,^{7,20,21,30,31} which is in an agreement with our study. The highest fold-change of 4.62 was detected for spot 961 between day 7 and day 14 after SNT surgery.

We also investigated the protein fold-changes in the left CeA of naive rats versus the left CeA of rats at day 7 and day 14 after SNT surgery (Figure 2), summarized in Table 3. Twenty-two matched spots showed significant difference, and 18 spots produced positive identification of proteins. We identified more than two proteins in 18 spots of the 2D gel by using nano-LC-MS/MS and peptide finger printing MALDI-TO2/TOF analyses, but identity of the proteins with the highest MASCOT score are discussed below. The highest fold-change (spot 638) was 3.69 between day 7 and day 14 after SNT surgery, and the protein was identified as ω -amidase NIT 2. The role of this protein is to remove potentially toxic intermediates. Spot 131 was identified as *N*-ethylmaleimide-

sensitive fusion protein and was down-regulated in SNT model rats at day 7 but up-regulated in SNT model rats at day 14. This protein is essential for numerous calcium triggered vesicle trafficking events. This protein functions as a molecular chaperone. It was previously reported that interaction between *N*-ethylmaleimide-sensitive factor and glutamate receptor is essential for fear memory formation in the CeA.^{20,21,32} Spot 730 was identified as *L*-isoaspartyl methyltransferase (PIMT), which catalyzes repair of *L*-isoaspartyl peptide bonds, a major source of protein damage. It was previously reported that PIMT knockout mice exhibit brain enlargement and fatal epileptic seizures.^{6,7,20,21} Spot 772 was identified as adenylate kinase, along with creatine kinase. Spot 899 was identified as vesicle-associated membrane proteins (VAMPs), which exhibit SNARE binding (soluble *N*-ethylmaleimide sensitive factor attached protein receptor) and were previously reported to be up-regulated during nerve regeneration.³³

Gene expression analysis of 14 significantly changed proteins in the right-side CeA and 19 proteins in the left-side CeA was performed by quantitative real-time PCR to further assess the regulation at the transcriptional level (Figures 3). The 2-fold change in these proteins occurred in rats between day 7 and day 14 after SNT surgery. Comparison of 14 transcript changes with the proteomics data (Figure 3a) revealed that all 14 changes were in the same direction by either analysis (13 lower and one higher expression in SNT model rats), whereas only 960

Table 3. Identified Proteins by Nano-LC-MS/MS from Spots Excised from 2D Gels^a

no.	spot no.	accession no. (SwissProt)	protein description	mascot score	MW	pI	seq cov (%)	fold change right-CeA	identified by
1	206	Q6P3V7	tetratricopeptide repeat protein GNN	29	150287	8.89	16	↓ (2.0)	nano-LC-Orbitrap Velos Pro MS
		Q99JD4	CLIP-associating protein 2	35	141463	8.79	15	↓ (2.0)	nano-LC-Orbitrap Velos Pro MS
2	210	P23348	anion exchange protein 3	35	135407	6.06	15	↓ (2.1)	nano-LC-Orbitrap Velos Pro MS
3	297	P15999	ATP synthase subunit α , mitochondrial	865	59754	9.22	38	↓ (2.0)	nano-LC-LTQ Orbitrap XL
		Q5XI77	annexin A11	821	54160	7.77	39	↓ (2.0)	nano-LC-LTQ Orbitrap XL
		Q6P6V0	glucose-6-phosphate isomerase	457	62827	7.89	22	↓ (2.0)	nano-LC-LTQ Orbitrap XL
4	300		<i>b</i>					↓ (2.0)	nano-LC-LTQ Orbitrap XL
5	411	P85515	α -centractin	171	42614	6.19	43	↓ (3.2)	nano-LC-LTQ Orbitrap XL
		Q01986	dual specificity mitogen-activated protein kinase kinase 1	163	43465	6.18	27	↓ (3.2)	nano-LC-LTQ Orbitrap XL
6	427		<i>b</i>					↑ (2.0)	
7	431	P50398	Rab GDP dissociation inhibitor α	1461	51074	5.00	69	↑ (2.9)	nano-LC-LTQ Orbitrap XL
		Q9Z0W5	protein kinase C and casein kinase substrate in neurons protein 1	102	50760	5.15	25	↑ (2.9)	nano-LC-LTQ Orbitrap XL
8	440		<i>b</i>					↑ (2.1)	nano-LC-LTQ Orbitrap XL
9	453	Q2LAM0	fatty acid 2-hydroxylase	29	42673	7.1	28	↓ (2.0)	nano-LC-LTQ Orbitrap XL
10	457	O54937	pyruvate dehydrogenase kinase isoform 4	32	46679	6.5	46	↓ (2.0)	nano-LC-LTQ Orbitrap XL
11	459	P07335	creatine kinase B-type	117	42725	5.42	52	↑ (2.9)	nano-LC-LTQ Orbitrap XL
		P82995	heat shock protein HSP 90- α	95	84815	5.01	39	↑ (2.9)	nano-LC-LTQ Orbitrap XL
		F1LTA7	uncharacterized protein (fragment)	69	42385	5.21	28	↑ (2.9)	nano-LC-LTQ Orbitrap XL
		D4A2Z9	uncharacterized protein	54	80700	4.91	31	↑ (2.9)	nano-LC-LTQ Orbitrap XL
		F1MSB6	uncharacterized protein (fragment)	42	76900	5.34	22	↑ (2.9)	nano-LC-LTQ Orbitrap XL
		D4AE04	uncharacterized protein	39	57300	5.22	25	↑ (2.9)	nano-LC-LTQ Orbitrap XL
		D3ZE66	uncharacterized protein	31	64700	5.12	19	↑ (2.9)	nano-LC-LTQ Orbitrap XL
12	536	P23565	α -internexin	197	56115	5.22	64	↑ (2.1)	nano-LC-LTQ Orbitrap XL
		P63039	60 kDa heat shock protein, mitochondrial	149	60955	6.18	44	↑ (2.1)	nano-LC-LTQ Orbitrap XL
		P63018	heat shock cognate 71 kDa protein	77	70871	5.52	46	↑ (2.1)	nano-LC-LTQ Orbitrap XL
		P61980	heterogeneous nuclear ribonucleoprotein K	62	50976	5.54	35	↑ (2.1)	nano-LC-LTQ Orbitrap XL
13	564		<i>b</i>					↑ (2.7)	nano-LC-LTQ Orbitrap XL
14	589	P34058	heat shock protein HSP 90- β	201	83281	5.03	49	↑ (3.0)	nano-LC-LTQ Orbitrap XL
		P82995	heat shock protein HSP 90- α	170	84815	5.01	38	↑ (3.0)	nano-LC-LTQ Orbitrap XL
		E9PSZ3	spectrin α chain, nonerythrocytic 1	48	284900	5.38	15	↑ (3.0)	nano-LC-LTQ Orbitrap XL
15	590	P14669	annexin A3	172	36363	5.96	13	↓ (2.9)	nano-LC-LTQ Orbitrap XL
		P20759	Ig γ -1 chain C region	39	36493	6.43	19	↓ (3.2)	nano-LC-LTQ Orbitrap XL
16	629	Q9Z2L0	voltage-dependent anion-selective channel protein 1	168	30756	8.62	48	↓ (3.5)	nano-LC-LTQ Orbitrap XL
		Q6PCU2	V-type proton ATPase subunit E 1	130	26128	8.44	35	↓ (3.5)	nano-LC-LTQ Orbitrap XL
17	643	P32851	syntaxin-1A	57	33067	4.9	13	↓ (2.5)	nano-LC-LTQ Orbitrap XL

Table 3. continued

no.	spot no.	accession no. (SwissProt)	protein description	mascot score	MW	pI	seq cov (%)	fold change right-CeA	identified by
18	663	F1LTA7	uncharacterized protein (fragment)	74	42385	5.67	5	↓ (3.0)	nano-LC-LTQ Orbitrap XL
19	700	P48500	triosephosphate isomerase	1575	26849	7.29	38	↓ (2.1)	nano-LC-LTQ Orbitrap XL
20	727	P63012	Ras-related protein Rab-3A	146	24970	4.9	22	↑ (2.1)	nano-LC-LTQ Orbitrap XL
		Q63942	GTP-binding protein Rab-3D	146	24290	4.8	18	↑ (2.1)	nano-LC-LTQ Orbitrap XL
21	728	O35244	peroxiredoxin-6	501	24819	5.64	25	↓ (2.0)	nano-LC-LTQ Orbitrap XL
22	733	<i>b</i>						↑ (2.1)	nano-LC-LTQ Orbitrap XL
23	773	P04906	glutathione S-transferase P	101	23439	7.44	12	↓ (2.0)	nano-LC-LTQ Orbitrap XL
		P48500	triosephosphate isomerase	35	26849	7.29	19	↓ (2.0)	nano-LC-LTQ Orbitrap XL
24	783	Q6P7Q4	lactoylglutathione lyase	236	20820	5.12	33	↓ (3.3)	nano-LC-LTQ Orbitrap XL
		P28073	proteasome subunit β type-6	154	25290	4.85	28	↓ (3.3)	nano-LC-LTQ Orbitrap XL
		O35509	Ras-related protein Rab-11B	134	24489	5.65	21	↓ (3.3)	nano-LC-LTQ Orbitrap XL
		Q5XI73	Rho GDP-dissociation inhibitor	128	23450	5.12	29	↓ (3.3)	nano-LC-LTQ Orbitrap XL
		Q00981	ubiquitin carboxyl-terminal hydrolase isozyme L1	120	25165	5.14	22	↓ (3.3)	nano-LC-LTQ Orbitrap XL
25	899	P63025	vesicle-associated membrane protein 3	100	11480	7.85	31	↓ (2.6)	nano-LC-LTQ Orbitrap XL
		Q63228	glia maturation factor β	89	16736	5.32	41	↓ (2.6)	nano-LC-LTQ Orbitrap XL
26	902	P10111	peptidyl-prolyl cis-trans isomerase A	124	17874	8.33	32	↓ (2.4)	nano-LC-LTQ Orbitrap XL
		Q9Z1B2	glutathione S-transferase μ 5	67	26629	6.74	15	↓ (2.4)	nano-LC-LTQ Orbitrap XL
27	929	Q63754	β -synuclein	135	14495	4.48	19	↓ (2.3)	nano-LC-LTQ Orbitrap XL
		Q6PEC4	S-phase kinase-associated protein 1	70	18831	4.4	20	↓ (2.4)	nano-LC-LTQ Orbitrap XL
28	939	<i>b</i>						↓ (2.5)	nano-LC-LTQ Orbitrap XL
29	940	P25113	phosphoglycerate mutase 1	478	28832	6.67	17	↓ (2.6)	nano-LC-LTQ Orbitrap XL
		P14604	enoyl-CoA hydratase, mitochondrial	377	31516	8.39	29	↓ (2.6)	nano-LC-LTQ Orbitrap XL
30	951	P54313	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β -2	564	37331	5.85	34	↑ (2.8)	nano-LC-LTQ Orbitrap XL
		P42123	L-lactate dehydrogenase B chain	254	36612	5.92	28	↑ (2.8)	nano-LC-LTQ Orbitrap XL
		P81799	N-acetyl-D-glucosamine kinase	213	37196	5.76	12	↑ (2.8)	nano-LC-LTQ Orbitrap XL
31	959	P07335	creatine kinase B-type	362	42725	5.39	27	↓ (2.6)	nano-LC-LTQ Orbitrap XL
		Q9JKB7	guanine deaminase, isoform CRA_b	221	50901	5.56	35	↓ (2.6)	nano-LC-LTQ Orbitrap XL
32	961	P04764	α -enolase	1147	47127	6.53	23	↓ (4.6)	nano-LC-LTQ Orbitrap XL
		P11275	calcium/calmodulin-dependent protein kinase type II subunit	506	54088	6.61	32	↓ (4.6)	nano-LC-LTQ Orbitrap XL
		P11730	calcium/calmodulin-dependent protein kinase type II subunit γ	28	59685	7.63	18	↓ (4.6)	nano-LC-LTQ Orbitrap XL
		P07323	γ -enolase	243	47141	4.91	54	↓ (4.6)	nano-LC-LTQ Orbitrap XL
		Q1KYT0	β -enolase	575	47130		34	↓ (4.6)	nano-LC-LTQ Orbitrap XL
33	967	<i>b</i>						↓ (2.2)	

^a11 cm IPG strip corresponding to the 1st group of neuropathic pain rat models. The central amygdala was excised from the right-side of rat brain.

^bNot detected.

Figure 3. Transcriptome versus proteome: (a) the right-side of CeA; (b) the left-side of CeA for group 1.

one protein showed opposite mRNA expression compared to protein expression in the left-side CeA (Figure 3b, NSF). The protein expression profiles from the right- and left-side CeA from sham rats and rats at days 3, 7, and 21 after SNT surgery were compared (group 2). The proteins were resolved on a 24 cm long IPG strip pH 3–11, and we loaded around 30 μ g of protein per gel. With this IPG strip, approximately 2100 spots were detectable per gel by silver staining. Two representative 2D gels for the left- and the right-side CeA as compared for sham versus days 3, 7, and 21 after SNT surgery are shown in Figures 4 and 5. Through differential analysis with Progenesis SameSpot software and comparison of the average spot intensity changes in the left-side CeA of sham rats versus rats at 3, 7, and 14 days after SNT surgery, we detected 18 spots as significantly regulated with a fold-change ≥ 2 . These spots were excised, and proteins from 15 spots were successfully identified by mass spectrometry. Each identified protein is listed Tables 4 and 5, linked to the corresponding 2D gel spot in Figures 4 and 5. Ten spots were consistently of decreasing intensity, two spots demonstrated changes between day 7 and day 21 after SNT surgery, and one spot demonstrated change between day 3 and day 7. Spot 459 was identified as Rab GDP dissociated inhibitor α and protein

kinase C and casein kinase substrate in neurons protein 1. The identities of these proteins were confirmed by nano-LC-MS/MS analysis and MALDI-TOF/TOF analysis. These proteins were also up-regulated in group 1 in the left-side CeA. Protein kinase C and casein kinase substrate in neurons protein 1 may play a role in vesicle formation and transport.^{1,5–7,20,21,34} Multiple proteins were identified from spot 1599, and these proteins were up-regulated in SNT models and also detected in group 1 as up-regulated proteins (Tables 1 and 2). The intensities of 14 spots were significantly different, and the protein identities of 13 spots were revealed by nano-LC-MS/MS and MALDI-TOF/TOF analyses when we compared 2D gels of the right-side CeA of sham rats versus those of the right-side CeA of rats at days 3, 7, and 21 after SNT surgery (Figure 5). Eight spots generated high intensities for days 3, 7, and 21 SNT models versus sham. More than two proteins were identified for five spots, which showed higher intensities versus the same spot from sham group. Some up-regulated proteins are as follows: ATP synthase subunit β , synaptosomal-associated protein 29 (spot 614), nucleoside diphosphate kinase (spot 1716), protein disulfide isomerase A3 (spot 507), and peroxiredoxin 5 (spot 1379). Peroxiredoxin 5 is a novel thioredoxin peroxidase recently identified in a variety of human

Figure 4. 2D gel of the left-side CeA. Arrows indicate spots with fold change ≥ 2 , when 2D gels of naive rats are compared to 2D gels of neuropathic pain model rats at days 3, 7, and 21 after SNT surgery. The first dimension, an isoelectric focusing (IEF), was performed with an immobilized pH gel (pH 3–11 NL) 24 cm long. The second dimension, SDS-PAGE, was performed with a 12% SDS polyacrylamide gel.

Figure 5. 2D gel of the right-side CeA. Arrows indicate spots with fold change ≥ 2 , when 2D gels of naive rats are compared to 2D gels of neuropathic pain model rats at days 3, 7, and 21 after SNT surgery. The first dimension, an isoelectric focusing (IEF), was performed with an immobilized pH gel (pH 3–11 NL) 24 cm long. The second dimension, SDS-PAGE, was performed with a 12% SDS polyacrylamide gel.

cells and tissues, which is considered to play an important role in oxidative stress protection mechanisms. L-Isoaspartyl/D-aspartyl O-methyltransferase (PIMT) was up-regulated in the right-side CeA of SNT model rats (spot 1591). The PIMT protein exhibits high activity in the brain, relative to other tissues, and is a putative protein repair enzyme, which converts L-isoaspartyl residues in damaged proteins to normal L-aspartyl residues. PIMT-deficient mice manifested neurodegenerative changes due to the accumulation of L-isoaspartate in the brain.³⁵ Spot 1670 was identified as protein L-isoaspartate (D-aspartate) O-methyltransferase and was also up-regulated in group 2. Five proteins were significantly down-regulated ($F \geq 2$, $p < 0.1$) in the right-side CeA of rats at days 3, 7, and 21

after SNT surgery, including phosphatidylethanolamine binding protein 1 in spot 1503, platelet-activating factor acetylhydrolase IB subunit β in spot 1088, and F-actin-capping protein subunit β in spot 1048; and for another two spots, 1675 and 819, more than two proteins were identified from the spot. Phosphatidylethanolamine-binding protein binds ATP, opioids, and phosphatidylethanolamine and was previously reported to be associated with memory and learning disorders.³⁶ Some proteins such as those contained in spots 1675 and 819 represent proteins of similar molecular weight with different pI values, which might be derived from post-translational modifications such as phosphorylation events.

Table 4. Identified Proteins by Nano-LC-MS/MS from Spots Excised from 2D Gels^a

no.	spots	fold	up/down-regulated	accession no. (SwissProt)	protein description	mascot	MW	pI	seq cov (%)	instrumentation
1	374	2.4	↓	P47942	dihydropyrimidinase-related protein 2	823	62278	6.33	14	nano-LC-LTQ Orbitrap XL
			↓	Q62950	dihydropyrimidinase-related protein 1	673	62499	6.64	44	nano-LC-LTQ Orbitrap XL
			↓	Q62951	dihydropyrimidinase-related protein 4	234	61617	6.3	34	nano-LC-LTQ Orbitrap XL
2	428	2.7	↓(D21); ↑(D3,D7)	P11980	pyruvate kinase isozymes M1/M2	456	58294	6.63	20	nano-LC-LTQ Orbitrap XL
			↓(D21); ↑(D3,D7)	Q4KM49	tyrosyl-tRNA synthetase, cytoplasmic	286	59420	6.57	20	nano-LC-LTQ Orbitrap XL
			↓(D21); ↑(D3,D7)	Q62952	dihydropyrimidinase-related protein 3	243	62327	6.04	11	nano-LC-LTQ Orbitrap XL
			↓(D21); ↑(D3,D7)	P19332	microtubule-associated protein tau	68	78801	5.95	19	nano-LC-LTQ Orbitrap XL
3	459	2.9	↑	P07335	creatine kinase B-type	117	42725	5.42	52	nano-LC-Orbitrap Velos Pro MS
			↑	P82995	heat shock protein HSP 90- α	95	84815	5.01	39	nano-LC-Orbitrap Velos Pro MS
			↑	F1LTA7	uncharacterized protein (fragment)	69	42385	5.21	28	nano-LC-Orbitrap Velos Pro MS
			↑	D4A2Z9	uncharacterized protein	54	80700	4.91	31	nano-LC-Orbitrap Velos Pro MS
			↑	F1M5B6	uncharacterized protein (fragment)	42	76900	5.34	22	nano-LC-Orbitrap Velos Pro MS
			↑	D4AE04	uncharacterized protein	39	57300	5.22	25	nano-LC-Orbitrap Velos Pro MS
			↑	D3ZE66	uncharacterized protein	31	64700	5.12	19	nano-LC-Orbitrap Velos Pro MS
4	498	2.5	↑	Q63537	synapsin-2	471	63702	8.70	25	nano-LC-LTQ Orbitrap XL
			↑	B2GV06	succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	384	56624	8.70	30	nano-LC-LTQ Orbitrap XL
			↑	Q6P6R2	dihydrolipoyl dehydrogenase, mitochondrial	305	54574	7.96	18	nano-LC-LTQ Orbitrap XL
5	599	2.5	↑	P50554	4-aminobutyrate aminotransferase, mitochondrial	269	57161	8.15	28	nano-LC-LTQ Orbitrap XL
6	630	2.3	↓	Q9QXQ0	α -actinin-4	419	42052	5.27	26	nano-LC-LTQ Orbitrap XL
			↓	P04764	α -enolase	417	47440	6.16	47	nano-LC-LTQ Orbitrap XL
			↓	P63269	actin, γ -enteric smooth muscle	278	42249	5.31	36	nano-LC-LTQ Orbitrap XL
			↓	P18298	S-adenosylmethionine synthase isoform type-2	166	44030	5.93	13	nano-LC-LTQ Orbitrap XL
			↓	Q6AYZ1	tubulin α -1C chain	112	50590	4.96	12	nano-LC-LTQ Orbitrap XL
			↓	Q3MIE4	synaptic vesicle membrane protein VAT-1 homologue	110	43319	6.17	28	nano-LC-LTQ Orbitrap XL
			↓	Q68FR6	elongation factor 1- γ	108	50371	6.31	17	nano-LC-LTQ Orbitrap XL
			↓	Q3KRE8	tubulin β -2B chain	104	50377	4.78	19	nano-LC-LTQ Orbitrap XL
			↓	P62909	40S ribosomal protein S3	99	26828	9.68	31	nano-LC-LTQ Orbitrap XL
7	679	2.8	↓	Q01986	dual specificity mitogen-activated protein kinase kinase 1	1208	43779	6.18	50	nano-LC-LTQ Orbitrap XL
			↓	P07936	neuromodulin	102	23703	4.61	20	nano-LC-LTQ Orbitrap XL
8	773	2.9	↓	Q5M7U6	actin-related protein 2	267	44990	6.30	42.4	nano-LC-LTQ Orbitrap XL
			↓	F1LPS8	transcriptional activator protein Pur- α	116	34976	6.07	17.4	nano-LC-LTQ Orbitrap XL
9	810	3.1	↑(D3,D7)		<i>b</i>					nano-LC-LTQ Orbitrap XL
10	933	2.5	↑		<i>b</i>					nano-LC-LTQ Orbitrap XL
11	993	4.0	↓	Q498U4	SAP domain-containing ribonucleoprotein	200	23647	6.30	23	nano-LC-LTQ Orbitrap XL

Table 4. continued

no.	spots	fold	up/down-regulated	accession no. (SwissProt)	protein description	mascot	MW	pI	seq cov (%)	instrumentation
12	1024	2.2	↓	B2RYW9	fumarylacetoacetate hydrolase domain-containing protein 2	488	34958	8.63	43	nano-LC-LTQ Orbitrap XL
13	1184	3.1	↑(D3); ↓(D7,D21)	Q6P0K8	junction plakoglobin	121	82490	5.75	23	nano-LC-LTQ Orbitrap XL
			↑(D3); ↓(D7,D21)	P60711	actin, cytoplasmic 1	107	42052	5.29	41	nano-LC-LTQ Orbitrap XL
			↑(D3); ↓(D7,D21)	F1LMV6	protein Dsp (desmoplakin)	88	334582	6.45	15	nano-LC-LTQ Orbitrap XL
14	1338	2.9	↓	Q642A5	DNA polymerase epsilon subunit 3	33	16674	4.39		MALDI-TOF/TOF
15	1342	2.0	↓	P04916	retinol-binding protein 4		23220	5.77	49	MALDI-TOF/TOF
16	1374	2.6	↓	P07632	Cu/Zn superoxide dismutase [<i>Rattus norvegicus</i>]		15912	6.34	21	nano-LC-LTQ Orbitrap XL
			↓	P37805	transgelin-3	27	22500	7.44		MALDI-TOF/TOF
17	1503	2.2	↓	P31044	phosphatidylethanolamine-binding protein 1	538	20902	5.48	75	nano-LC-LTQ Orbitrap XL
18	1591	2.5	↑	P22062	protein L-isoaspartate (D-aspartate) O-methyltransferase	120	24683	7.14	45	MALDI-TOF/TOF
19	1599	2.6	↑	P50398	Rab GDP dissociation inhibitor α	1461	51074	5.00	69	nano-LC-LTQ Orbitrap XL
		2.6	↑	Q9Z0W5	protein kinase C and casein kinase substrate in neurons protein 1	102	50760	5.15	25	nano-LC-LTQ Orbitrap XL
			↑	Q5XI34	protein Ppp2r1a	195	65323	5.11	53	nano-LC-Orbitrap Velos Pro MS
			↑	P24090	α -2-HS-glycoprotein, isoform CRA_b		38002			nano-LC-LTQ Orbitrap XL
20	1664	2	↑	P11980	isoform M2 of pyruvate kinase isozymes M1/M2	330	58294	6.63	45	nano-LC-LTQ Orbitrap XL
		2	↑	P12928	pyruvate kinase isozymes R/L	64	62504	6.45	19	nano-LC-LTQ Orbitrap XL
		2	↑	P62630	elongation factor 1- α 1	63	50424	9.10	13	nano-LC-LTQ Orbitrap XL
		2.0	↑	O88758	potassium voltage-gated channel subfamily S member 1		54914	7.66	45	MALDI-TOF/TOF
21	1724	2.4	↑		<i>b</i>					nano-LC-LTQ Orbitrap XL

^a24 cm IPG strip corresponding to the 2nd group of neuropathic pain rat models. The central amygdala was excised from the left-side of rat brain.
^bNot determined.

Two spots (1591 and 1503) were identified with more than 2-fold change in the left- versus right-side CeA.

The differentially expressed proteins were annotated in public repository data by Gene Ontology (GO) terms. Proteins are described by GO terms as biological process, cellular components, and molecular function. Figure 6 represents proteins annotated according to their molecular functions compared to an entire rat proteome. Approximately 83% of all identified proteins had protein binding function, 76% catalytic activity, 31% nucleotide binding, 28% metal ion binding, and 7% transporter activity. GO biological process annotation analysis revealed that more than 78% of proteins were involved in metabolic processes, regulation of biological processes, and response to stimulus. Approximately 22% of all identified proteins were involved in transport, development, and cell organization and biogenesis.

Membrane Protein Mass Spectrometry-Based Proteomics

Membrane proteins are key regulators of crucial processes including the passage of information and substances between cells and mediating activities such as hormone action and nerve impulses. Membrane proteins are scarcely soluble in the buffers used during isoelectric focusing due to the exposure of hydrophobic amino acids on their quaternary structure, leading to a partial extraction of certain species or complete absence from the final gel. Therefore, we prefractionated proteins with sodium carbonate washes and obtained crude membrane

fractions. We utilized two approaches in this work: (a) 1D-PAGE combined with nano-LC-MS/MS analysis for identification of high molecular weight proteins and (b) stable isotope dimethyl labeling and quantitation of membrane proteome in SNT models.

1D-SDS-PAGE Identification of Membrane Proteins. We utilized 1D-SDS-PAGE methodology combined with nano-LC-MS/MS analysis for the identification of the membrane proteins. The membrane fraction consisting of membrane and membrane associated proteins from sham animals was prepared by differential centrifugation. Samples were solubilized in a buffer containing 4% SDS. The presence of the strong detergent SDS ensured practically complete solubilization of membrane proteins. Five bands of approximately equal size covering the molecular weight range of 80 to 230 kDa were cut out from SDS-PAGE (Figure S3) and subjected to in-gel digestion with trypsin. The resultant peptides were extracted and analyzed by nano-LC-MS/MS. To identify the proteins present, the MS/MS spectra were submitted to the Mascot algorithm. Mascot then identified the tryptic peptides by matching their MS/MS spectra against *in silico* generated theoretical spectra from the database. A single run on the LTQ-Orbitrap instrument identified approximately 800 proteins. The combination of our sample preparation procedure with high resolution MS analysis yielded a large number of brain-specific proteins. These include neuro-

Table 5. Identified Proteins by Nano-LC-MS/MS from Spots Excised from 2D Gels^a

spots	fold	up/down-regulated	accession	protein description	mascot	MW	pI	Seq. Cov. (%)	Instrumentation
459	2.5	↑	KCRB_RAT	creatine kinase B-type	117	42725	5.42	52	nano-LC-LTQ Orbitrap XL
		↑	HS90A_RAT	heat shock protein HSP 90-α	95	84815	5.01	39	nano-LC-LTQ Orbitrap XL
		↑	FILTA7_RAT	uncharacterized protein (fragment)	69	42385	5.21	28	nano-LC-LTQ Orbitrap XL
		↑	D4A2Z9_RAT	uncharacterized protein (fragment)	54	80700	4.91	31	nano-LC-LTQ Orbitrap XL
		↑	F1M5B6_RAT	uncharacterized protein (fragment)	42	76900	5.34	22	nano-LC-LTQ Orbitrap XL
		↑	D4AE04_RAT	uncharacterized protein	39	57300	5.22	25	nano-LC-LTQ Orbitrap XL
		↑	D3ZE66_RAT	uncharacterized protein	31	64700	5.12	19	nano-LC-LTQ Orbitrap XL
507	3.1	↑	PDIA3_RAT	protein disulfide-isomerase A3	360	57044	5.88	31	nano-LC-LTQ Orbitrap XL
		↑	PRS4_RAT	26S protease regulatory subunit 4	101	49325	5.87	40	nano-LC-LTQ Orbitrap XL
		↑	SEPT9_RAT	septin-9	80	65169	7.14	25	nano-LC-LTQ Orbitrap XL
		↑	PRS4_RAT	26S protease regulatory subunit 4	111	49325	5.87	29	nano-LC-Orbitrap Velos Pro MS
		↑	ACTB_RAT	actin, cytoplasmic 1	109	42052	5.29	40	nano-LC-Orbitrap Velos Pro MS
		↑	DCIL1_RAT	cytoplasmic dynein 1 light intermediate chain 1	100	56985	6.13	11	nano-LC-Orbitrap Velos Pro MS
510	2.4	↑	G6PI_RAT	glucose-6-phosphate isomerase	595	62958	7.38	37	nano-LC-LTQ Orbitrap XL
		↑	SYN2_RAT	synapsin-2	323	63702	8.73	25	nano-LC-LTQ Orbitrap XL
		↑	CAP1_RAT	adenylyl cyclase-associated protein 1	173	51899	7.16	12	nano-LC-LTQ Orbitrap XL
		↑	SCOT1_RAT	succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	112	56624	8.70	9	nano-LC-LTQ Orbitrap XL
534	2.8	↑	Q5XI34_RAT	protein phosphatase 2 (formerly 2A)	401	65323	4.75	25	nano-LC-LTQ Orbitrap XL
		↑	2AAB_RAT	serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A β	367	66006	4.73	35	nano-LC-LTQ Orbitrap XL
		↑	TBA1B_RAT	tubulin α-1B chain	230	50152		43	nano-LC-LTQ Orbitrap XL
		↑	GDIA_RAT	guanosine diphosphate dissociation inhibitor 1, isoform CRA_a	127	50538	4.75	23	nano-LC-LTQ Orbitrap XL
614	2.0	↑	ATPB_RAT	ATP synthase subunit β, mitochondrial	120.0	56354	4.92	18	MALDI-TOF/TOF
		↑	SNP29_RAT	synaptosomal-associated protein 29	51.0	29071	5.12	17	MALDI-TOF/TOF
		↑	G3V6D3_RAT	ATP synthase subunit β	656.21	56345	5.27	77	nano-LC-Orbitrap Velos Pro MS
		↑	TBB5_RAT	tubulin β-5 chain	219.82	49671	4.89	54	nano-LC-Orbitrap Velos Pro MS
		↑	G3V7C6_RAT	protein Tubb2c	218.9	49831	4.89	48	nano-LC-Orbitrap Velos Pro MS
		↑	ENOG_RAT	γ-enolase	69.57	47141	5.16	45	nano-LC-Orbitrap Velos Pro MS
		↑	GRP78_RAT	78 kDa glucose-regulated protein	130.54	72347	5.16	58	nano-LC-Orbitrap Velos Pro MS
630	2.6	↓	ACTN4_RAT	α-actinin-4	419	42052	5.27	26	nano-LC-LTQ Orbitrap XL
		↓	ENOA_RAT	α-enolase	417	47440	6.16	47	nano-LC-LTQ Orbitrap XL
		↓	ACTH_RAT	actin, γ-enteric smooth muscle	278	42249	5.31	36	nano-LC-LTQ Orbitrap XL
		↓	METK2_RAT	S-adenosylmethionine synthase isoform type-2	166	44030	5.93	13	nano-LC-LTQ Orbitrap XL
		↓	TBA1C_RAT	tubulin α-1C chain	112	50590	4.96	12	nano-LC-LTQ Orbitrap XL
		↓	VAT1_RAT	synaptic vesicle membrane protein VAT-1 homologue	110	43319	6.17	28	nano-LC-LTQ Orbitrap XL
		↓	EF1G_RAT	elongation factor 1-γ	108	50371	6.31	17	nano-LC-LTQ Orbitrap XL
		↓	TBB2B_RAT	tubulin β-2B chain	104	50377	4.78	19	nano-LC-LTQ Orbitrap XL

Table 5. continued

spots	fold	up/down-regulated	accession	protein description	mascot	MW	pI	Seq. Cov. (%)	Instrumentation
700	2.8		TPIS_RAT	triosephosphate isomerase		26849	7.29	23.0	nano-LC-LTQ Orbitrap XL
773	↓		ARP2_RAT	actin-related protein 2	267	44990	6.30	42.4	nano-LC-LTQ Orbitrap XL
	↓		FILPS8_RAT	transcriptional activator protein Pur- α	116	34976	6.07	17.4	nano-LC-LTQ Orbitrap XL
810				^b					nano-LC-LTQ Orbitrap XL
819	2.6	↓	NDUAA_RAT	NADH dehydrogenase [ubiquinone] 1 α subcomplex subunit 10, mitochondrial	542	40753	7.64	52.0	nano-LC-LTQ Orbitrap XL
	↓		TALDO_RAT	transaldolase	76	37608	6.57	19.0	nano-LC-LTQ Orbitrap XL
	↓		ALDOC_RAT	fructose-bisphosphate aldolase C	76	39658	6.67	18.0	nano-LC-LTQ Orbitrap XL
	↓		ICAIL_RAT	islet cell autoantigen 1-like protein	43	48644	5.01		nano-LC-LTQ Orbitrap XL
848	2.1	↑	AKIA1_RAT	alcohol dehydrogenase [NADP+]	340	36506	7.36		MALDI-TOF/TOF
1048	2.0	↓	CAPZB_RAT	F-actin-capping protein subunit β	813	30952	5.69	63.2	nano-LC-LTQ Orbitrap XL
1088	3.9	↓	PAIB2_RAT	platelet-activating factor acetylhydrolase IB subunit β	121	25736	5.57	13.5	nano-LC-LTQ Orbitrap XL
	↓		H2A1C_RAT	histone H2A type 1-C	88	14097	11.05	21.5	nano-LC-LTQ Orbitrap XL
1184	2	↑		^b					
1379	2.1	↑	PRDX5_RAT	peroxiredoxin-5, mitochondrial	177	22507	8.94	45.0	nano-LC-LTQ Orbitrap XL
1503	2.1	↓	PEBP1_RAT	phosphatidylethanolamine binding protein 1, isoform CRA_b		20801	5.58		nano-LC-LTQ Orbitrap XL
1591	2.1	↑	PIMT_RAT	protein L-isoaspartate (α -aspartate) O-methyltransferase	120	24683	7.14	45.0	nano-LC-LTQ Orbitrap XL
1599	2.6	↑	GDIA_RAT	Rab GDP dissociation inhibitor α	1461	51074	5.00	69.1	nano-LC-LTQ Orbitrap XL
	↑		PACN1_RAT	protein kinase C and casein kinase substrate in neurons protein 1	102	50760	5.15	25.0	nano-LC-LTQ Orbitrap XL
	↑		Q5XI34_RAT	protein Ppp2r1a	195	65323	5.11	53.3	nano-LC-Orbitrap Velos Pro MS
	↑		FETUA_RAT	α -2-HS-glycoprotein, isoform CRA_b		38002			nano-LC-LTQ Orbitrap XL
1664	2.0	↑	KCNS1_RAT	potassium voltage-gated channel subfamily S member 1	22	54914	7.66	45.0	MALDI-TOF/TOF
	↑		KPYM_RAT	isoform M2 of pyruvate kinase isozymes M1/M2	330	58294	6.63	45.0	nano-LC-Orbitrap XL
	↑		KPYR_RAT	pyruvate kinase isozymes R/L	64	62504	6.45	4.9	nano-LC-Orbitrap XL
	↑		EFIA1_RAT	elongation factor 1- α 1	63	50424	9.1	2.4	nano-LC-Orbitrap XL
1670		↑		^b					nano-LC-LTQ Orbitrap XL
1675	2.4	↓	PDIA3_RAT	protein disulfide-isomerase A3	751	57044	5.88	49.1	nano-LC-LTQ Orbitrap XL
	↓		PRS4_RAT	26S protease regulatory subunit 4	295	49325	5.87	18.0	nano-LC-LTQ Orbitrap XL
	↓		PPP5_RAT	serine/threonine-protein phosphatase 5	108	57507	5.84	9.6	nano-LC-LTQ Orbitrap XL
	↓		PTMA_RAT	prothymosin α	79	12375	3.75	13.0	nano-LC-LTQ Orbitrap XL
	↓		HSPB1_RAT	heat shock protein β -1	62	22936	6.12	4.9	nano-LC-LTQ Orbitrap XL
1716	3.1	↑(D21); ↓(D3, D7)		rCG57843, isoform CRA_a	1	17283			nano-LC-LTQ Orbitrap XL
	↑(D21); ↓(D3, D7)		NDKB_RAT	nucleoside diphosphate kinase	1	17283	7.57		nano-LC-LTQ Orbitrap XL
	↑(D21); ↓(D3, D7)		KPYM_RAT	pyruvate kinase isozymes M1/M2	6	57818	7.50		nano-LC-LTQ Orbitrap XL

^a24 cm IPG strip corresponding to the 2nd group of neuropathic pain rat models. The central amygdala was excised from the right-side of rat brain. ^bNot determined.

Figure 6. Breakdown of differentially expressed proteins according to molecular function for groups 1 and 2. Differentially expressed proteins at days 7 and 14 after SNT surgery were uploaded to Protein Center and annotated according to Gene Ontology terms.

Figure 7. Mass spectrum of the triplex stable isotope dimethyl labeled peptide. Light, intermediate, and heavy labeled peptides shifted by 28.03, 32.06, and 36.08 m/z , corresponding to 56.06, 64.11, and 72.15 Da as the peptide ion is doubly charged. This peptide is dimethyl-labeled on one site showing m/z difference of 2.01.

transmitter receptors such as glutamate and GABA receptors and also ion channels such as sodium and potassium channel proteins. The identified glutamate receptors represent NMDA, glutamate receptor epsilon-1, and metabotropic glutamate receptor 3. Voltage-dependent ion channels identified include potassium voltage-gated channel subfamily A member 3, voltage-gated calcium channel $\alpha 2/\delta$ -1 subunit, sodium channel protein type 2 subunit α , Nav 1.2, and potassium voltage-gated channel subfamily A member 4.

Membrane Protein Quantification Using Stable Isotope Dimethyl Labeling Mass Spectrometry Technology. We utilized the triplex stable isotope dimethyl

labeling method performed at the peptide level in this work. Triplex stable isotope dimethyl labeling is based on the reaction of peptide primary amines with formaldehyde to generate a Schiff base that is rapidly reduced by the addition of cyanoborohydride to the mixture.¹⁷ A mass increase of 32 Da per primary amine is achieved using deuterated formaldehyde ("intermediate" label). By combining deuterated and ^{13}C -labeled formaldehyde with cyanoborodeuteride, we achieved the third label with a mass increase of 36 Da ("heavy" label). We employed on-column stable isotope dimethyl labeling to differentially label three samples to allow simultaneous quantitative LC-MS analysis. We used trypsin as the enzyme

for digestion of proteins; this cleaves C-terminally of lysine and arginine residues. We performed the trypsin digestion in buffer without primary amines in triethylammonium bicarbonate (TEAB) buffer, because this method might be prone to contaminating and interfering components remaining after sample preparation. These components might include primary amine-containing molecules such as ammonium bicarbonate or Tris. As formaldehyde will also react with these amines, these compounds affect the labeling efficiency. Following desalting the peptide sample before labeling using self-made μ C18 columns, peptides of the different samples were on-column labeled separately with either of the dimethyl isotopomers, and the final eluates from three μ C18 StageTips were combined. This protocol was optimized for a sample amount of 15 μ g using 3 mg of 3 cm^3 C₁₈ SepPak stationary phase packed into a 10 μ L tip.^{17,37,38} In MS, the different stable isotopically labeled peptides are measured, and these peptides are recognized by the known mass difference between them. When the tryptic peptides are labeled with this method, most peptides differ in mass by either 4 Da (when cleaved after an arginine residue, only the N terminus is labeled) or 8 Da (when cleaved after a lysine residue, both the N terminus and the lysine residues are labeled) between the light and intermediate and between the intermediate and heavy label. Our samples of which peptides were differentially stable isotope dimethyl labeled by the on-column protocol were separated by a 290 min gradient on a nano-LC reversed phase column. This way, more peptides were identified and quantified as the total MS time was increased and peptides were better resolved. We employed relatively long columns and small particle sizes (50 μm , 1.8 μm) to facilitate deep sampling of the proteome. We found the combination of a 50 cm column and nearly 5 h gradient to be a good combination for standard use. We utilized a Q Exactive mass spectrometer, which is capable of determining peptide precursor charge states and fully resolving all peaks with high-resolution (for example, resolution more than 90000 was achieved at m/z 524). Therefore, the m/z of the labeled peptides was measured with this high resolution, and peptide ions were selected for fragmentation (MS/MS) to determine the amino-acid sequence. After the LC-MS/MS run, these tandem MS spectra were matched against theoretical spectra obtained from a protein sequence database using Mascot algorithm, which was capable of identifying differentially labeled stable isotope dimethyl-labeled peptides. Quantification was achieved by comparing the intensities of the differentially labeled peptides at MS level, integrated with their extracted ion chromatograms using Proteome Discovery software.

The adult rat CeA is a small brain region, which possibly contains about 1 mg of wet tissue and around 80–100 μg of total protein mass. Therefore, in depth analysis of the CeA membrane proteome of one single rat required a precise analytical method. Before applying this technology to SNT samples, we analyzed control samples to validate the protocol. A BSA digest (5 μg of protein per label) was dimethyl labeled in a 1:1:1 light/intermediate/heavy ratio. A m/z shift compared with the nonlabeled peptide (m/z 927.6) of, respectively, 28.03, 32.06, and 36.08 was found for the light, intermediate, and heavy peptide ions, respectively (Figure 7). As the peptide ions are doubly charged, these m/z shifts correspond to mass increments of, respectively, 56.06, 64.11, and 72.15 Da. This is consistent with the fact that the peptide has two sites available to be labeled, the N terminus and the

lysine residue. Complete labeling was obtained as no peak at the m/z corresponding to unlabeled peptides was observed in the dimethyl-labeled sample. The sample was analyzed in triplicate by nano-LC-MS/MS. The LC-MS/MS files were combined and analyzed by Proteome Discoverer 1.3 Thermo Scientific. Mascot identified peptides, which account for 95% of the sequence BSA (Swiss-Prot-UniProt, P02769), and none of nonlabeled peptides were identified upon database searching with Mascot.

To validate the protocol further, we prepared membrane fractions from sham rats and neuropathic pain model rats at day 7 and 21 after SNT surgery using Na₂CO₃ washes, and the resulting membrane pellets were solubilized in 0.2% ProteaseMax Surfactant in 50 mM TEAB. ProteaseMax Surfactant (molecular weight 451.51 Da) was a good detergent to solubilize membrane proteins. It is designed to degrade over the course of a trypsin digestion reaction and completely degraded with an addition of 5% acetic acid, just before on-column dimethyl labeling, producing a hydrophilic zwitterionic species (molecular weight 139.17 Da) and neutral hydrophobic species (molecular weight 238.36 Da), which do not interfere with mass spectrometry analysis at that concentration. After protease digestion, this sample (~15 μg of protein) was split in three equal portions, and the peptides were on-column differentially stable isotope dimethyl labeled in a 1:1:1 light/intermediate/heavy ratio and mixed. We use approximately 5 μg of protein per label. In the first instance for checking the quality of our samples, we analyzed this sample by MALDI-TOF/TOF. A ratio of 1:1:1 was obtained for ~97% of triplex isotope dimethyl labeled peptides. The labeled peptide mixtures were then loaded separately onto the autosampler of the nano-LC system (Ultimate 3000) and analyzed in an automated manner by nano-LC-MS/MS on a Q Exactive mass spectrometer. The addition of three dimethyl labels makes the peptide more hydrophobic, thereby increasing its retention on the nano-C18 column. A slight separation was observed between the differently labeled species of the same peptide. Using Proteome Discoverer software and stringent filtering criteria as described in Materials and Methods, we quantified around 650 proteins per LC-MS/MS run. We combined three technical nano-LC-MS/MS runs of the same sample, and we quantified around 1300 proteins. The performance of the dimethyl labeling experiment was found to be around 80% of the identified proteins quantified.

Around 3 μg of membrane proteins from the CeA of sham rats and rats at days 3, 7, and 21 after SNT surgery were reduced and alkylated and in-solution digested with trypsin, and the resulting peptides were purified on μ C18 Stage Tips and triplex dimethyl labeled with light, intermediate, and heavy tags, followed by 1:1:1 mixing. We used two biological samples. Samples were analyzed by nano-LC-MS/MS using the 5 h gradient. We analyzed each sample in triplicate by LC-MS/MS. Proteome Discoverer software incorporated with Mascot was used to identify and quantify peptides and proteins. The combination of the data generated from mixture 1 samples allowed the cumulative quantification of more than 1300 protein groups, 6000 merged proteins, 4500 peptides, and 110000 PSMs (FDR < 0.5%), and around 950 proteins were quantified per ratio of either heavy/light or intermediate/light. Next, we filtered our data sets to keep only proteins found in both biological samples and calculated their median isotopic ratios (heavy (SNT day 21)/light (sham); intermediate (SNT day 3)/light (sham); intermediate (SNT day 7)/light (sham)),

and duplicate ratios generated from mixture 1 and 2 samples of heavy (SNT day 21)/light (sham) were combined (Figure 8).

Figure 8. Venn diagram shows quantified proteins in mixture 1 and mixture 2 samples for heavy/light ratio.

Doublecortin (DCX) was quantified as one of the proteins significantly up-regulated in membrane fractions of the CeA of rats at 3, 7, and 21 days after SNT surgery compared to sham rats, with sequence coverage of around 50%, and 6 peptides were taken for quantification analysis. Figure 9 shows MS/MS spectrum of m/z 724.9130 corresponding to doubly charged peptide NVNPNWSVNVK, which was dimethyl labeled on two sites.

DCX is microtubule-associated protein required for initial steps of neuronal dispersion and cortex lamination during cerebral cortex development.³⁹ This protein may act by competing with the putative neuronal protein kinase DCLK1 in binding to a target protein and may in that way participate in a signaling pathway that is crucial for neuronal interaction before and during migration, possibly as part of a calcium ion-dependent signal transduction pathway. It also found to participate along with PAFAH1B1/LIS-1 in a distinct overlapping signaling pathway that promotes neuronal migration.

Validation of Altered Expression Levels by Western Blotting

Western blotting revealed that the expression level of DCX was localized in the CeA tissue lysate and was present in equal amount in the CeA of sham and neuropathic pain model animals (Figure 10b). Consistent with the mass spectrometry findings, DCX was detected in the membrane fractions of the CeA in neuropathic model animals but not in sham ones (Figure 10a). Immunoblot analysis showed a significant increase in the levels of DCX in membrane fraction in the CeA of animals at day 21 after SNT surgery confirming mass spectrometry data.

Figure 10. Western blots of the CeA. (a) Protein content of DCX in membrane fractions of the CeA from sham and neuropathic pain rats at days 3, 7, and 21 after SNT surgery. Equal amounts of protein were loaded onto the gel followed by SDS-PAGE gel electrophoresis and immunoreaction. (b) Protein content of whole tissue lysate shows that DCX in the CeA did not differ in sham and neuropathic pain models.

Immunohistochemistry of DCX in the CeA of Sham and SNT Model Rats

We used a triple labeling fluorescence experiment performed with DCX and 58K Golgi protein antibodies plus DAPI to localize DCX in the CeA of sham animal. Figure 11 shows

Figure 11. In the CeA of sham rats, DCX (green) is found along with 58K Golgi protein and colocalizes to the Golgi apparatus as shown by colocalization of a Golgi marker and DCX.

triple staining using 58K Golgi maker and DAPI of a 10 μ m brain section from sham animal, co-stained for DCX. DCX

Figure 9. MS/MS spectrum of m/z 724.9130 $[M + H]^{2+}$ ion corresponding to peptide NVNPNWSVNVK, which was dimethyl-labeled on two sites.

SNT day 7

SNT day 21

Figure 12. Representative immunohistochemical images from the CeA of rats at days 7 and 21 after SNT surgery demonstrating colabeling with DCX (green) and ATPase (red), respectively. The sections are counterstained with DAPI nuclear marker.

Figure 13. Expression changes of selected genes in the CeA of rats at day 21 after SNT surgery by quantitative real-time PCR and by 2D electrophoresis as compared to sham. Red bar represents cDNA fold change normalized to yWHAZ, and blue bars represent protein fold change.

1273 displayed colocalization with 58K Golgi protein indicating the
1274 presence of DCX in the Golgi apparatus. Triple immuno-
1275 fluorescent labeling was repeated for rats at 7 and 21 days after
1276 SNT and revealed that DCX and the Golgi marker were not
1277 colocalized. We also studied localization of DCX at the plasma
1278 membranes. Colocalization of DCX with $\alpha 1$ Na,K-ATPase
1279 subunit and DAPI was examined in the CeA of sham rats and
1280 rats at 7 and 21 days after SNT surgery. $\alpha 1$ Na,K-ATPase
1281 subunit antibody was used as a plasma membrane marker. We
1282 observed that DCX is present in processes of the CeA in these
1283 SNT model animals and colocalizes with the plasma
1284 membrane marker, $\alpha 1$ Na,K-ATPase (Figure 12), but not in
1285 the CeA of sham. Together, the DCX distribution by
1286 immunohistochemistry showed that DCX is expressed in
1287 processes in the CeA of rats 7 and 21 days after SNT surgery.
1288 This suggests that doublecortin contributes to the neuronal
1289 plastic changes in the CeA in neuropathic pain models.

**Validation of Differentially Expressed Proteins by
Real-Time PCR for Group 2 SNT Models**

To verify the reliability of the proteomics analysis, we selected
26 genes of the central amygdala differentially changed at day
21 after SNT surgeries for quantitative real-time PCR analysis.
The mRNA levels of 19 genes followed the same up-
regulation trend after SNT surgery in comparison to sham,
supporting proteome data ($p < 0.05$), while for five genes
mRNA expression levels were down-regulated in rats at day 21
after SNT surgery. It is likely that this relates to mRNA-
independent post-translational modification, trafficking, and
degradation, as a large number of modification sites and
domains. However, this could also be due to qPCR
experiments being carried out using cDNA extracted from
whole CeA. Figure 13 shows the proteome and transcriptome
fold changes in neuropathic pain rats at day 21 after SNT
surgery.

1307 ■ DISCUSSION

1308 We know that peripheral nerve injury could lead to altered
1309 regulation of a large number of molecules. Several gene array
1310 studies and proteomics studies have been directed to
1311 investigate the genes regulated in CNS following nerve injury.
1312 A recent report by Wu et al. showed effects of electro-
1313 acupuncture stimulation on protein levels in amygdala of
1314 complete Freund's adjuvant-induced inflammatory pain model
1315 rats using iTRAQ labeling.⁴⁰ They showed more than 100
1316 altered proteins in the painful condition, and this proves the
1317 hypothesis that plastic changes occur in amygdala in pain
1318 conditions, albeit they used the entire amygdala rather than
1319 focusing on the CeA. In this study, we performed proteomics
1320 investigation of neuropathic CeA using the spinal nerve
1321 transection model in which animals developed signs of
1322 neuropathy at days 7 and 14 (group 1) and at days 3, 7, and
1323 21 (group 2) post-transection of L5 spinal nerve. In both
1324 groups, we found CeA proteome changes in a complex
1325 asymmetrical and time-related manner following neuropathy.

1326 We identify more than 50 proteins in the right-side CeA and
1327 around 45 proteins in the left-side CeA in group 1. Those
1328 differentially expressed proteins are involved in physiological,
1329 metabolic, and cellular processes and development processes.
1330 In summary, many identified proteins involved in neural
1331 plasticity/neurotransmitter release were either up-regulated in
1332 the right-side CeA after SNT surgery (e.g., cofilin-1, syntaxin-
1333 1A) or down-regulated in the control side (e.g., F-actin-
1334 capping protein α -2, transgelin-3). Multiple subtypes of Ras-
1335 related protein Rab (involved in protein transport) were up-
1336 regulated in the right-side CeA after SNT surgery. Both α - and
1337 γ -enolase (suggested to be involved in cell survival) were
1338 down-regulated in the right-side CeA after SNT surgery, while
1339 protein DJ-1 (protects neurons against oxidative stress and cell
1340 death) was up-regulated.

1341 We observed decrease in mitogen-activated protein kinase in
1342 the right-side CeA. Mitogen-activated protein kinases,
1343 including extracellular signal-regulated kinase, mediate plasti-
1344 city in the amygdala and hippocampus. Creatine kinase B was
1345 decreased after nerve injury in both sides of CeA in this study.
1346 Creatine has been found to reduce glutamate levels and to
1347 exhibit neuroprotective properties.⁴¹ Therefore, the down-
1348 regulation of creatine kinase B might be particularly important
1349 for the development and maintenance of neuropathic pain. γ -
1350 Enolase was up-regulated in rats at day 7 but down-regulated
1351 in rats at day 14 after SNT surgery. Neuronal γ -enolases exist
1352 in the synaptic plasma membrane⁴² and may interact with
1353 creatine kinase and pyruvate kinase, and they were identified in
1354 the right-side CeA but not in the left-side. Calcium/
1355 calmodulin-dependent protein kinase type II subunit α (α -
1356 CaMKII) was down-regulated the right-side CeA in neuro-
1357 pathic pain rats. α -CaMKII is a critical contributor to the
1358 spontaneous and ongoing component of tissue-injury evoked
1359 persistent pain. Dual specificity mitogen-activated protein
1360 kinase kinases (MAPKs) play critical roles in regulating neural
1361 plasticity and inflammatory responses. Vesicle-fusing ATPase
1362 N-ethylmaleimide-sensitive fusion protein (NSF) was down-
1363 regulated in the left-side CeA 14 days after surgery but up-
1364 regulated at day 7. This protein was reported to be involved in
1365 central sensitization in the spinal cord through GluR2 subunit
1366 composition switch after inflammation. Protein L-isoaspartyl
1367 methyltransferase was up-regulated in neuropathic pain rats at
1368 day 7 but down-regulated at day 14 after surgery, with no

1369 difference between the left and right CeA. This protein
1370 catalyzes repair of L-isoaspartyl peptide bonds, a major source
1371 of protein damage under physiological conditions.

1372 We concluded that asymmetric changes in the proteome
1373 were observed with the right-side CeA dominating. Also,
1374 protein change in the right-side CeA was higher at day 14 after
1375 surgery, and differences between the left- and right-side CeA
1376 proteome change were observed in all groups.

1377 Even through group 2 consisted of different days after SNT
1378 surgeries, we identified around 20 proteins that were up- or
1379 down-regulated in the left- and the right-side CeA as in group
1380 1, for example, γ -enolase, creatine kinase, pyruvate kinase M1
1381 and M2, glycolytic enzymes, transgelin-3, peroxiredoxin-6, dual
1382 specificity mitogen-activated protein kinase kinase 1, pyruvate
1383 kinase, phosphatidylethanolamine-binding protein 1, protein L-
1384 isoaspartyl methyltransferase, triosephosphate isomerase, heat
1385 shock proteins 60 and 90, and ATP synthase subunit α . These
1386 regulated identified proteins function in diverse pathways such
1387 as inflammation, cellular metabolism, defense against oxidation
1388 and neuronal cell death, and signal transduction (membrane-
1389 associated proteins). This could be explained that in the
1390 nervous system, nerve injury and stress can cause oxidative
1391 damage to the cytoskeleton and membrane structures. In
1392 addition, significant energy production is required for protein
1393 expression and cellular defense mechanisms. This is supported
1394 by the increased expression of proteins involving antioxidative
1395 processes, neuronal integrity and glycolysis/gluconeogenesis
1396 observed in this work. The expression of thirty-five differential
1397 proteins was further examined using quantitative real-time
1398 PCR. Overall, we found that changes were similar to those
1399 identified by 2D electrophoresis, and only a few gene
1400 expression levels were not correlated to mRNA levels. It is
1401 likely that this relates to mRNA-independent post-translational
1402 modification, trafficking, and degradation, as there are a large
1403 number of modification sites and domains.

1404 In conclusion, we observed the regulation of a large
1405 population of proteins in cellular defense mechanisms and
1406 antioxidation. The up-regulation of many proteins in the actin
1407 cytoskeletal organization strongly suggests the concept that
1408 stabilization of actin cytoskeleton is one of the priorities in a
1409 fight against injury or stress.^{43,44} Nearly half of the proteins
1410 that we identified in this study have been shown to be similarly
1411 regulated at the mRNA levels or proteins levels after other
1412 types peripheral nerve injury, validating our experimental
1413 procedures.

1414 There are several notable limitations to this study. First,
1415 proteome quantification by silver staining has several issues as
1416 a standard quantification (e.g., poor linear dynamic response,
1417 nonspecific staining of nucleic acids and lipopolysaccharides,
1418 nonsensitivity in detecting certain proteins).⁴⁵ In order to
1419 mitigate these issues, each sample was run in triplicate and the
1420 relative quantification of a spot was calculated as the mean of
1421 its relative volumes; the mean coefficient of variation (CV) for
1422 these relative volumes was 20% ($\pm 5\%$). Based on this finding,
1423 only a protein expression ratio of less than 1.5 can be
1424 empirically deemed experimental variation. Therefore, only
1425 those protein spots that were up-regulated or down-regulated
1426 by at least 2.0-fold with $p < 0.1$ were considered significantly
1427 differentiated. The most important drawback of gel-based and
1428 mass spectrometry-based proteomics is that it is not possible to
1429 analyze the entire proteome. Proteins displayed in a single 2D
1430 gel represent only a portion of the proteins in a sample, and
1431 low-abundance proteins such as regulatory proteins and

receptors are often not detected by conventional staining. Membrane proteins are not readily amenable to 2D electrophoresis due to their poor solubility in standard protein extraction solutions.^{46,47} Therefore, our study could only identify a limited set of differentially expressed proteins after SNT surgery.

Quantification of Membrane Proteome of the CeA from Neuropathic Pain Model Animals

Membrane proteins are key regulators of crucial processes including the passage of information and substances between cells and mediating activities such as hormones action and nerve impulses. More than 30% of the mammalian genome encodes membrane proteins. These proteins are challenging to study but essential to understand, as they represent two-thirds of drug targets. Mapping of membrane proteome and identification of drug targets requires methods for tissue extraction, protein processing, and sensitive and specific analysis. Taking advantage of our established membrane purification method for microscale identification and quantification of membrane proteins in this work and recent development in high resolution and high accuracy mass spectrometry, we performed an in-depth comparative analysis of membrane proteomes of sham versus neuropathic pain model rats. The CeA contains around 100 μ g of total protein. Therefore, we had a limited amount of membrane proteins from neuropathic pain model rats, but despite the mass limitations, we were able to identify as many as 1300 proteins and quantify around 950 proteins from a single biological analysis. Synaptoporin, syntaxin, sodium channel subunit 1, and metabotropic glutamate receptor were up-regulated at day 3 after SNT surgery. Our findings also support an impairment of synaptic function in the CeA, especially for components of soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptor (SNARE) function. Syntaxin 6, which was unregulated, is a cellular receptor for transport vehicle, which inhibits SNARE function and thereby controls neurotransmitter release. The endocannabinoid system, which consists of the cannabinoid receptors referred to as CB1 and CB2⁴⁸ and endogenous ligands, can regulate pain perception.⁴⁹ CB1 receptors are associated with the peripheral and central nervous systems, and CB1 agonists have been shown to reduce hyperalgesia in animal models of inflammatory,⁵⁰ neuropathic,⁵¹ capsaicin,⁵² and cancer pain.⁵³ CB1 receptor was up-regulated in this study. Neurochodrin was unregulated in SNT model rats, and it was previously shown that neurochodrin seems to interact with phosphatidic acid, which is a metabolite related to phospholipid metabolism. DCX was up-regulated in the membrane fraction of the CeA in SNT model rats. DCX protein is associated with immature neuronal phenotypes and elevated neuroplasticity in the adult brain, and evidence from the rodent studies suggests that stress may modify its expression in amygdala, and it was found to be increased in the CeA.⁵⁴ DCX is mainly found in immature neurons during development of the nervous system and is believed to play an important role in neurogenesis, neuro-migration, and axon growth. This is why DCX has been widely used as a marker for immature neurons. Interestingly, DCX has been used as a marker for adult neurogenesis as well, typically in hippocampal dentate gyrus and the subventricular zone.^{55,56} Such adult neurogenesis in those regions of brain apparently implicates the impact of neurogenesis in memory formation and learning and the olfactory system. However, recent

findings suggest tissues that have previously not been strongly linked to neurogenesis have upregulated DCX, including dorsal root ganglia.⁵⁷ Thus, DCX seems to be involved not only in neurogenesis in developing brain but also in reformation or rewiring of neurons in multiple tissues such as dorsal root ganglia and central amygdala.

We found significant increase of DCX in the membrane fraction without changes in total amount of the protein in the CeA. DCX is known to associate with microtubules, but it is also reported that DCX associates with membrane bound actin via spinophilin.⁵⁸ Spinophilin is known as an actin binding protein, but it also binds to some membrane proteins such as G-protein coupled receptors. DCX may link microtubules to actin and other membrane-bound proteins via spinophilin and promote axonal growth in renetworking of neurons within and beyond the CeA in chronic pain conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.9b00805>.

Nissl staining image showing rat amygdala, right-side vs left-side CeA of naive rats and rats at day 7 and day 14 after SNT surgery, 2D gel of the left-side CeA, and entire gel image for the Western blot (GAPDH) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Kenji Okuse — Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, U.K.; orcid.org/0000-0001-8860-9352; Phone: +44-0207-5945742; Email: k.okuse@imperial.ac.uk

Authors

Kersti Karu — Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, U.K.
Richard S. Swanwick — Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, U.K.
Amparo Novejarque-Gadea — Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London SW10 9NH, U.K.
Ana Antunes-Martins — The Wolfson Centre for Age-Related Diseases, King's College London, London SE1 1UL, U.K.
Benjamin Thomas — Central Proteomics Facility, Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.
Eiji Yoshimi — Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, U.K.
William Foster — Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, U.K.
Min Fang — Public Health England, London SE1 8UG, U.K.
Stephen B. McMahon — The Wolfson Centre for Age-Related Diseases, King's College London, London SE1 1UL, U.K.
David L. H. Bennett — Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford OX3 9DU, U.K.
Andrew S. C. Rice — Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London SW10 9NH, U.K.

Complete contact information is available at:

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1553 Notes

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