

## RESEARCH ARTICLE

# The significance of biochemical and molecular sample integrity in brain proteomics and peptidomics: Stathmin 2-20 and peptides as sample quality indicators

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Comparisons of transcriptional and translational expression in normal and abnormal states are important to reach an understanding of pathogenesis and pathophysiology. Maintaining the biochemical, molecular, and structural sample integrity is essential for correct sample comparisons. We demonstrate that both proteins and neuropeptides, including their PTMs, are subjected to massive degradation in the brain already 1 min postmortem. Further, markers for determining the integrity and status of a biological sample were identified. The protein fragment stathmin 2-20 correlated well with the general level of postmortem degradation and may serve as a sample quality indicator for future work, both in animal and human postmortem brains. Finally, a novel method for preventing degradation of proteins and peptides in postmortem tissue is presented using rapid and uniform conductive heat transfer on tissue prior to the actual sample preparation procedures, which enables the relatively low-abundant neuropeptides to remain intact, minimizes degradation of proteins by proteolysis, and conserves the PTMs of the neuropeptides.

Received: February 9, 2007  
Revised: September 3, 2007  
Accepted: September 4, 2007

**Keywords:**

Brain / Peptide degradation / Peptidomics / Protein degradation / Protein phosphorylation

## 1 Introduction

With the development of genomic and proteomic technologies, the use of postmortem tissue in human and animal neurosciences has received increased interest. Comparisons of transcriptional and translational expression in normal and

abnormal states are important to reach an understanding of pathogenesis and pathophysiology [1]. Hence, for the successful application of these technologies, it is of outmost importance that the biochemical, molecular, and structural integrity of the sample is appropriately maintained [1].

While it is generally expected that the postmortem interval may significantly affect the preservation of some brain neuropeptides and catecholamines, it is commonly thought that proteins are relatively stable postmortem [2–4]. However, protein recoveries from human postmortem tissue have displayed high variability. In recent reports, the postmortem changes observed for proteins have mainly concerned structural proteins and enzymes, appearing between 6 and 48 h postmortem [5]. Moreover, it has been suggested that factors from the premortem period and, in particular, the agonal

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**Abbreviations:** CLIP, corticotropin-like intermediate lobe peptide; LTQ, linear trap quadrupole; MAPK, mitogen-activated protein kinase; Q-TOF, quadrupole time-of-flight; UniProtKB, Universal Protein Resource Knowledgebase

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state and the rapidity of death, may play major roles in determining the postmortem condition of the sample [6–9]. It would therefore be of importance to determine the condition and quality of postmortem tissue, for example, through the quantification of a biological marker in such sample.

In a previous report [10], we presented a universal peptide display, a peptidomic approach, utilizing online nano-scale capillary RP LC and ESI MS (nano-LC ESI-MS) for the simultaneous analysis of peptides from different rat brain regions. However, these analyses mainly detected high levels of peptide fragments from abundant proteins, not likely reflecting the *in vivo* composition of the neuropeptides. The relatively short postmortem interval from sacrifice to tissue preparation (3 min) was sufficient to produce a large amount of protein fragments which masked the MS detection of neuropeptides at low concentration. The proteins and peptides extend over at least ten orders of magnitude in concentration in the brain, thus limiting the methods for their simultaneous detection. More recently, we utilized a sample preparation technique including *in vivo* protein denaturation by focused microwave irradiation in combination with the peptidomic approach [11]. The identified peptides in this study were either known endogenous neuropeptides or novel endogenous peptides. Peptide fragments from high abundant proteins were not present, demonstrating that protein degradation was prevented by rapid heating and inhibition of proteolytic activity postmortem.

The present study was designed to display rapid, within minutes postmortem changes of susceptible peptides and proteins in the brain and to identify markers for determining the postmortem quality of a sample. In addition, we investigated an alternative sample inactivation method which was compared to focused microwave irradiation for enabling the analysis of fresh or snap-frozen samples.

By analyzing the peptides and proteins of brain samples subjected to different postmortem time intervals, it was found that the degradation of a number of proteins and neuropeptides started immediately. A large number of protein fragments were detected in the peptidomic analysis only 1 min postmortem. PTMs of endogenous peptides and proteins, such as phosphorylations, also disappeared rapidly postmortem. However, a small number of biologically active endogenous peptides and neuromodulators were relatively stable up to 10 min postmortem. We also identified a protein fragment in both human and mouse brains from stathmin (stathmin 2-20) that correlated with the general level of postmortem degradation of biological samples and may serve as a quality indicator in future work.

## 2 Materials and methods

### 2.1 Experimental subjects and sample handling

The present study examined tissue with different postmortem intervals from mouse (C57/BL6, male) and human

brains. All animal procedures were approved by the local animal ethics committee. The human postmortem brain tissue were obtained from the Stanley Foundation Brain Bank [12].

In a first experiment 16 mice were randomly divided into four groups (Table 1). The first group was sacrificed by focused microwave irradiation (Muromachi Kikai, Tokyo, Japan), 1.4 s at 4.5–5 kW (control group). Three groups of animals were sacrificed by cervical dislocation and kept in room temperature (22°C) at different time intervals, 1, 3, and 10 min, respectively, and the brains were subsequently irradiated by focused microwaves. Striatum, hypothalamus, and cortex were thereafter rapidly dissected out and stored at –80°C. The hypothalamus was used for analysis using the peptidomic approach, the cortex for 2-D DIGE analysis, and striatum for the study of the phosphorylation state of mitogen-activated protein kinase (MAPK).

In a second experiment four mice were sacrificed by cervical dislocation and the head of the animals were immediately cooled in liquid nitrogen. The striatum (two striata from each brain hemisphere;  $n = 8$ ) was rapidly dissected out on dry ice before freezing at –80°C. The samples were then prepared using two different protocols. In the first procedure the striatum samples from one hemisphere ( $n = 4$ ) were immediately heated to 95°C using a denaturing device for biological samples (Denator AB, Gothenburg, Sweden). The instrument uses a combination of vacuum, pressure, and heat to induce a rapid and homogeneous heating of the sample, resulting in inactivation of protease activity. In the second procedure the remaining striatum samples from the other hemisphere ( $n = 4$ ) were prepared at 4°C. The tissue from this experiment was used for peptidomics and Western blot analysis.

In a third experiment we studied human brain tissue samples (six males and six females), the cingulate cortex from healthy normal individuals, from postmortem time intervals between 7 and 62 h (six samples with postmortem intervals 7–18 h, four males, two females; and six samples with longer postmortem intervals 50–62 h, two males, four females). The samples were divided into two groups with comparable postmortem intervals. Six samples were prepared using the rapid heat transfer inactivation instrument (Denator) for 20 s at 95°C, and the remaining six samples were prepared at 4°C. The human brain samples were analyzed by the peptidomic approach.

### 2.2 Peptidomic sample preparations

The brain tissues were suspended in cold extraction solution (0.25% acetic acid) and homogenized by microtip sonication (Vibra cell 750, Sonics & Materials, Newtown, Connecticut) to a concentration of 0.2 mg tissue (wet weight)/ $\mu\text{L}$ . The suspension was centrifuged at  $20\,000 \times g$  for 30 min at 4°C. The protein- and peptide-containing supernatant was transferred to a centrifugal filter device (Microcon YM-10, Millipore, Bedford, MA) with a nominal molecular weight limit of 10 000 Da,

**Table 1.** Experimental procedures for the mouse postmortem time study

Experiment	Group	Sacrificing method	Postmortem interval	Brain area	Protocol	Methodology
1	1	Focused MW	1.4 s	Hypothalamus	Dissection, –80°C storage, sample preparation	Peptidomics
				Cortex	Dissection, –80°C storage, sample preparation	DIGE
				Striatum	Dissection, –80°C storage, sample preparation	Western blot
	2	Cervical dislocation	1 min	Hypothalamus	Focused MW, dissection, –80°C storage, sample preparation	Peptidomics
				Striatum	Focused MW, dissection, –80°C storage, sample preparation	Western blot
	3	Cervical dislocation	3 min	Hypothalamus	Focused MW, dissection, –80°C storage, sample preparation	Peptidomics
				Striatum	Focused MW, dissection, –80°C storage, sample preparation	Western blot
	4	Cervical dislocation	10 min	Hypothalamus	Focused MW, dissection, –80°C storage, sample preparation	Peptidomics
				Cortex	Focused MW, dissection, –80°C storage, sample preparation	DIGE
				Striatum	Focused MW, dissection, –80°C storage, sample preparation	Western blot
2	1	Cervical dislocation	3 s	Striatum	Snap-frozen, liq. N <sub>2</sub> , dissection, –80°C storage, heated 95°C, sample preparation	Peptidomics
			3 s	Striatum	Snap-frozen, liq. N <sub>2</sub> , dissection, –80°C storage, heated 95°C, sample preparation	Western blot
	2	Cervical dislocation	3 s	Striatum	Snap-frozen, liq. N <sub>2</sub> , dissection, –80°C storage, thawed 4°C, sample preparation	Peptidomics
3	1	–	7, 12, 18, 50, 60, 62 h	Cingulate cortex	–80°C storage, heated 95°C, sample preparation	Peptidomics
	2	–	8, 10, 12, 56, 60, 60 h	Cingulate cortex	–80°C storage, thawed 4°C, sample preparation	Peptidomics

In experiment 1, a group of mice (controls) were sacrificed by focused microwave irradiation, and three other groups were sacrificed by cervical dislocation and subsequently irradiated by focused microwaves at the postmortem intervals 1, 3, and 10 min, prior to freezing and storage at –80°C and sample preparation. In experiment 2, mice were sacrificed by cervical dislocation and the brains immediately snap-frozen in liquid N<sub>2</sub>. The samples were then either treated with the rapid heating instrument or were thawed to 4°C and prepared for peptidomics analysis (MW, microwave; N<sub>2</sub>, nitrogen; liq., liquid).

and centrifuged at 14 000 × *g* for 45 min at 4°C. Finally, the peptide filtrate was frozen and stored at –80°C until analysis.

### 2.3 Relative quantification and identification of endogenous peptides using nano-LC MS

Five microliters of peptide filtrate (equivalent to 1.0 mg wet weight brain tissue) was injected onto a fused silica capillary column (75 µm id, 15 cm length, NAN75-15-03-C18PM; LC Packings, Amsterdam, The Netherlands). The particle bound sample was desalted by an isocratic flow of buffer A (0.25% acetic acid in water) for 35 min and eluted during a 60 min gradient from buffer A to B (35% ACN in 0.25% acetic acid), delivered using a nano-LC system (Ettan MDLC, GE Healthcare, Uppsala, Sweden). The eluate was directly infused to an ESI quadrupole TOF (Q-TOF) mass spectrometer (Q-ToF-2, Micromass, Manchester, UK) at a flow rate of approximately 120 nL/min for analysis.

Data acquisition from the ESI Q-TOF instrument was performed in continuous mode and mass spectra were collected at a frequency of 3.6 GHz and integrated into a single spectrum each second. Mass spectra were collected in the *m/z* range of 300–1000 Da. ESI Q-TOF MS data collected during the 60 min chromatographic peptide separation was exported as a text file using DataBridge (MassLynx 3.4, Micromass) to DeCyder MS 1.0 (GE Healthcare) for visualization and detection of peak profiles. This program aligns the different LC-MS profiles and by integrating the ion

intensities over the eluting peptide enables peptide-specific intensity comparisons. The peaks were counted, matched, and semiquantitatively compared between the different samples.

### 2.4 Endogenous peptide identification using Q-TOF linear trap quadrupole (LTQ) MS/MS

The peptides were identified by matching masses from deconvoluted peaks (DeCyderMS 1.0, GE Healthcare), via DataBridge (MassLynx 3.4, Micromass) to the SwePep database v. 2006-02-15 containing 4180 nonredundant peptide sequences [13] (www.sweep.com). The proposed identities were subsequently confirmed by manual sequencing of CID ESI-tandem MS (MS/MS) spectra.

Sequence information was obtained in data-dependent MS/MS acquisition mode. The intensity threshold for MS to MS/MS switching was set to 12 ion counts. The collision chamber was filled with argon with the inlet pressure set to about 15 psi. The collision energy was ramped from 23 to 31 eV in 5 s. CID spectra were collected in the *m/z* range of 40–1200. Spectral visualization and interpretation was made in PepSeq (BioLynx, MassLynx 3.4, Micromass) where the proposed peptide sequences from the SwePep database search were used. Each peptide identity was confirmed by visual inspection of the *de novo* spectrum.

The peptide filtrates were also analyzed using nano-LC LTQ MS/MS (Thermo Electron, San Jose, CA) and infused

as described above. The spray voltage was 1.8 kV, the capillary temperature was 160°C, and 35 units of collision energy were used to obtain fragment spectra. Four MS/MS spectra of the most intense peaks were obtained following each full-scan mass spectrum (Xcalibur 1.4 SR1). The dynamic exclusion feature was enabled to obtain MS/MS spectra on coeluting peptides. Raw LTQ data were converted to dta files by Xcalibur and put together by an inhouse developed script to MASCOT generic files. The corresponding endogenous peptides were identified by correlating the MS/MS spectra to the *mus musculus* subdatabase of the minimally redundant Universal Protein Resource Knowledgebase (UniProtKB)/Swiss-Prot database 10 431 entries (release 48.8) [14] using MASCOT 2.1 [15]. The search parameters were as follows: partial oxidation of methionine (+16 Da). Precursor-ion mass tolerance of 1.5 Da, peptide mass tolerance of 1.5 Da, and fragment ions tolerance of 0.8 Da. No enzyme was specified to be used. The criteria for positive identification of a peptide was a MASCOT score >51. MASCOT score is based on the absolute probability (*P*) that the observed match between the experimental data and the database sequence is a random event. The criteria are chosen to be 0.05 (5% chance of a false positive), this translates into a threshold score of 51.

## 2.5 Proteomic sample preparation

The cortical brain areas were lysed by sonication on ice in ice-cold lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM TrisCl) at pH 8.5 and centrifuged at  $14\,000 \times g$  at 4°C for 30 min. The protein concentration of each homogenate was determined using Protein Determination Reagent Plus One 2-D Quant Kit (GE Healthcare).

## 2.6 DIGE

Lyophilized cyanine dyes (CyDye DIGE, Cy2, Cy3, Cy5 minimal dyes; GE Healthcare) were reconstituted in DMF (Aldrich) to a concentration of 400 pm/μL. For each homogenate, 50 μg of protein was labeled with 400 pmol of either Cy3 or Cy5. Cy2 was used to label the internal standard, which was prepared from pooled aliquots of equal amounts of the samples. The pooled standard was labeled in bulk in sufficient quantity to include a standard on every gel. The experiment was performed in duplicate comparing three individual samples from the 0 min and from the 10 min preparation, respectively. The six samples from the two groups were labeled in a random manner according to standard DIGE experimental design [16]. Prior to IEF the labeled samples were mixed and added to an equal volume of  $2 \times$  sample buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/mL DTT, 4% Pharmalyte 3-10 (GE Healthcare).

## 2.6.1 Analytical gels

All 2-D separations were performed using standard GE Healthcare 2-D PAGE apparatus and reagents. In brief, 24 cm Immobiline DryStrips pH 3–10 NL were used for the first dimension separation by anodic cup-loading technique. IEF was performed using the Ettan IPGphor II for a total of 48 kV · h. Following IEF, strips were equilibrated with reducing buffer containing 6 M urea, 1% w/v SDS, 30% v/v glycerol, 37.5 mM Tris-HCl pH 6.8, 30 mM DTT for 15 min and subsequently equilibrated with alkylating buffer containing 6 M urea, 1% w/v SDS, 30% v/v glycerol, 37.5 mM Tris-HCl pH 6.8, 240 mM iodoacetamide for a further 15 min. Second-dimension SDS polyacrylamide gel electrophoresis was performed using 1.0 mm thick 12.5% SDS polyacrylamide gels in low-fluorescence glass cassettes, using an Ettan DALT *twelve* Separation unit. Modified Laemmli buffer (0.2% SDS) was used as running buffer at 17 W *per* gel with constant voltage until the dye front reached the bottom of the gel.

## 2.6.2 Preparative gels

Mass spectrometric protein identifications were carried out from two preparative gels. Prior to gel casting, two reference markers were attached to the bind-silane treated glass plate as position references for spot picking. Five hundred micrograms of unlabeled pooled standard was loaded using the in-gel rehydration technique and separated as described above for analytical gels. The gels were stained using SYPRO Ruby Protein Gel Stain (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

## 2.7 DIGE imaging and analysis

Gel images were generated by scanning the gels in a Typhoon 9400 laser scanner (GE Healthcare) using the following settings: Cy2 (488 nm excitation laser and 540 BP40 nm emission filter), Cy3 (532 nm excitation laser and 580 BP30 emission filter), and Cy5 (633 nm excitation laser and 670 BP30 nm emission filter). The preparative gel was scanned with 457 nm excitation laser and 610 BP30 emission filter. The differential in-gel analysis (DIA) module of the DeCyder analysis software (V 5.02; GE Healthcare) was used for codetection of spots in the three spectrally resolved images for each gel using 2500 as estimated number of spots for detection. The DIA module automatically quantitates spot protein abundance for each image and expresses these values as ratios allowing direct comparison of corresponding spots. Matching between gels utilizing the common internal standard was performed in DeCyder biological variation analysis (BVA) module. This enables quantitative comparison and statistical analysis of samples between gels based on the relative change of sample to its internal standard [16]. Two groups representing the 0 min and the 10 min samples, respectively, were created to allow statistical analysis (Student's *t*-test) between the groups. Criteria for selecting spots

with changes in abundance were set to: absolute value of average fold ratio  $\geq 1.5$ ;  $p$ -value  $\leq 0.05$ ; the spot should also be detected in all gel images and in both experimental replicates.

### 2.7.1 Automated spot picking

The protein spots in the analytical gels that met the defined statistical requirements (see above) were matched to the preparative gel spot maps. Spot picking, trypsin digestion, and peptide extraction was performed automatically in the Ettan Spot Picker and the Ettan Digester (GE Healthcare) according to standard protocols. Briefly, the plugs were washed in 50 mM ammonium bicarbonate and dried prior to digestion with trypsin in 20 mM ammonium bicarbonate (37°C for 70 min). The peptide fragments were extracted with 50% v/v ACN in 0.1% v/v TFA for 60 min and then dried. The digests were mixed with 5  $\mu$ L 0.25% acetic acid in water.

## 2.8 Protein identification

The trypsin digest was injected and desalted on a precolumn (PepMapT, LC Packings) at a flow rate of 10  $\mu$ L/min of buffer A (0.25% acetic acid in water) for 10 min. A 15 cm, 75  $\mu$ m id fused silica emitter (Proxeon Biosystems, Odense, Denmark) packed with Reprosil-Pur C18-AQ 3  $\mu$ m resin (Dr. Maisch, Ammerbuch-Entringen, Germany) was used as the analytical column. The samples were analyzed during a 40 min gradient from 3 to 60% buffer B (84% ACN in 0.25% acetic acid) delivered using a nano-LC system (Ettan™ MDLC, GE Healthcare). The eluate was directly infused to an LTQ mass spectrometer (Thermo Electron) similar to described above.

The data-dependent acquisition and dynamic exclusion feature of the LTQ provided sequence information of most of the eluting peptides. Raw LTQ data were converted to dta files by Xcalibur 1.4 SR1 and put together by an inhouse developed script to MASCOT generic files. The corresponding proteins were identified by correlating the MS/MS spectra to the *mus musculus* subdatabase of the minimally redundant UniProtKB/Swiss-Prot database 10 431 entries (release 48.8) [14] using MASCOT 2.1 [15]. The search parameters were as follows: partial oxidation of methionine (+16 Da), and cysteine (+57 Da). Precursor-ion mass tolerance of 1.5 Da, peptide mass tolerance of 1.5 Da, and fragment ions tolerance of 0.8 Da. Trypsin was specified as used enzyme allowing for one missed cleavage. The criteria for positive identification of a peptide was a MASCOT score  $>30$ . MASCOT score is based on the absolute probability ( $p$ ) that the observed match between the experimental data and the database sequence is a random event. The criteria are chosen to be 0.05 (5% chance of a false positive), this translates into a threshold score of 30. A protein is accepted when four or more peptides from a single protein are proposed.

## 2.9 Western blotting to detect phosphorylations of proteins

Experiments for quantifying phosphorylations on MAPK were carried out as previously described [17]. Frozen striatal tissue samples were sonicated in boiling 1% SDS and boiled for 10 min. Small aliquots of the homogenate were retained for protein determination with a BCA-kit (Pierce, Rockford, IL, USA), using BSA as a standard. Equal amounts of protein (15  $\mu$ g) were loaded onto 4–20% TGI acrylamide gels (Invitrogen, Stockholm, Sweden) and the proteins were separated by SDS-PAGE and transferred to NC membranes (0.2  $\mu$ m, Immobilon-PSQ, Millipore, Stockholm, Sweden). The membranes were immunoblotted using commercially available polyclonal antibodies against phospho-Thr202/Tyr205-MAPK (1:500; Chemicon, Temecula, CA, USA), MAPK (1:1000). Antibody binding was revealed by incubation with goat antirabbit HRP-linked IgG (Pierce Europe, Oud Beijerland, the Netherlands) and the ECL immunoblotting detection system (GE Healthcare). Chemiluminescence was detected by autoradiography using DuPont NEN autoradiography film (Sigma, Sweden). The levels of phosphorylated and total MAPK were quantified by densitometry using National Institutes of Health IMAGE 1.61 software.

## 3 Results

### 3.1 Peptidomic analysis of postmortem samples

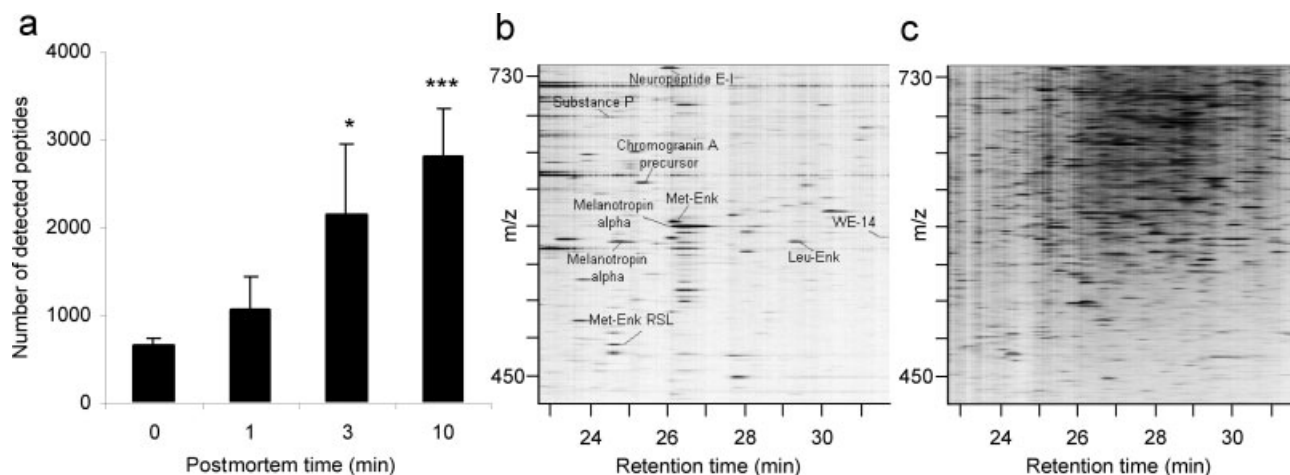
#### 3.1.1 Postmortem time effect on the number of detected peptides

Mice were sacrificed by focused microwave irradiation (control group) as previously described [11]. Striatal brain samples from such mice displayed an average of  $664 \pm 101$  distinct MS peaks using nano-LC ESI-MS (Fig. 1). Other groups of mice were thereafter killed by cervical dislocation and exposed to microwave irradiation postmortem. After 1 min postmortem delay peptides from degrading high abundant proteins were detected. In this group, the number of peptides increased to  $1064 \pm 568$  compared to control. Three minutes postmortem the number of peptides was  $2156 \pm 1109$  and after 10 min  $2805 \pm 654$  peptides were detected. The identities of the degraded proteins can be found in Table 1 of Supporting Information.

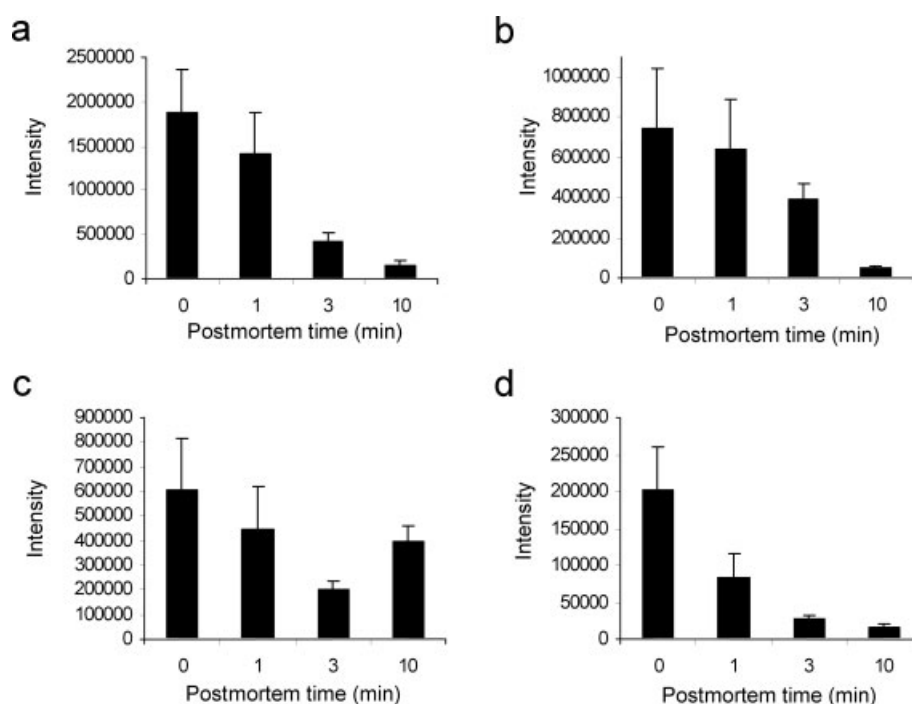
#### 3.1.2 Postmortem time effect on neuropeptides and their relative levels

In the control group, a number of known neuropeptides were detected and identified by MS/MS. These consisted of known neuropeptides, hormones, and potential new biologically active peptides. Altogether 23 neuropeptides, hormones, and potential biologically active peptides were





**Figure 1.** Postmortem time effect on the number of detected peptides in the mouse hypothalamus. (a) The number of detected peptides by nano-LC ESI MS increase with postmortem time. Y-axis display number of detected peptides ( $\pm$  SD,  $n = 4$  in each group). The 3 and 10 min postmortem groups differed significantly from the control group (\* $p < 0.05$ , \*\*\* $p < 0.001$ ,  $t$ -test). (b, c) Two-dimensional graphs of the control group and 10 min postmortem group, respectively.



**Figure 2.** Postmortem time effect on the relative levels of neuropeptides, (a) proopiome-lanocortin-derived peptide, (b) beta endorphin, (c) CLIP, and (d) phosphorylated CLIP.

identified (see Table 2 of Supporting Information). In this study generally, most biologically active peptides, including enkephalins, neuropeptide EI, and beta-endorphin were present in higher levels in the control group compared to 1, 3, and 10 min postmortem (Figs. 2a and b). A few peptides, *i.e.*, thymosin  $\beta$ -4 and  $\beta$ -10, little SAAS and neurotensin were stable through the 10 min time course. Several novel potential biologically active peptides were also detected.

### 3.1.3 Postmortem time effects on PTMs on peptides

The corticotropin-like intermediate lobe peptide (CLIP) was identified both with and without a phosphate group at Ser-154. The levels of the unphosphorylated CLIP decreased over postmortem time, 1 and 3 min, but increased after 10 min (Fig. 2c). The phosphorylated form of CLIP also decreased over postmortem time. One minute postmortem the level was >50% lower compared to the control group (Fig. 2d).

### 3.1.4 Discovery of an *in situ* peptide marker for sample integrity determination

A peptide fragment which is derived from the abundant and ubiquitously expressed phosphoprotein stathmin (stathmin 2-20) was detected at increasing levels with increasing post-mortem time in the mouse study. The peptide was not detected in the control group. This peptide, which is highly conserved between species, may therefore serve as a marker for the determination of the quality of a brain sample. The stathmin fragment showed a distinctive and reproducible postmortem temporal degradation profile (Fig. 3a).

### 3.1.5 Peptidomic analysis of human brain tissue samples

The stathmin 2-20 peptide was also identified in human postmortem brain tissue. The detected relative levels of the stathmin 2-20 peptide were lower in those samples treated with the rapid conductive heat inactivation instrument (Denator AB; Fig. 3b,  $p < 0.001$ , paired  $t$ -test). The number of detected peptides was similarly lower in those samples treated with the inactivation instrument compared to the samples not treated with the instrument and prepared at 4°C. No significant sex-specific differences were found.

## 3.2 Protein analysis of postmortem samples

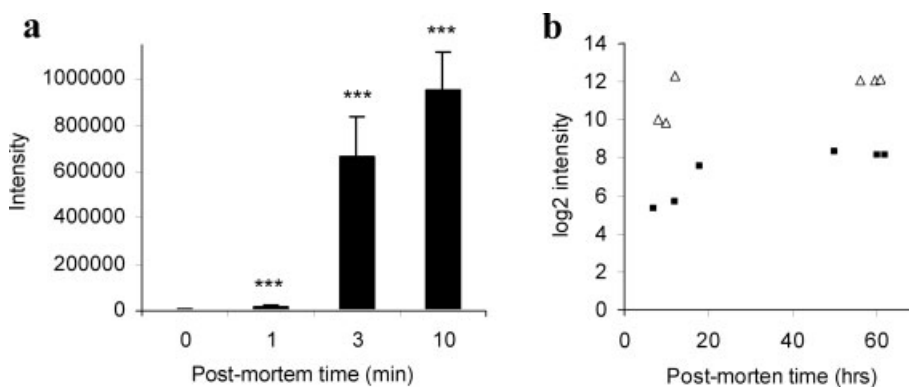
### 3.2.1 Postmortem time effect on protein abundance

Cortical brain tissue was obtained from the focused irradiated microwave control group (0 min postmortem) and the 10 min postmortem group. Protein changes were compared between these two groups by DIGE technology with a mixed-sample internal standard and codetection. Well-resolved cortical tissue protein spot maps were obtained resolving typically above 1500 proteins (Fig. 4). DeCyder image analysis revealed 53 spots with a significant absolute abundance

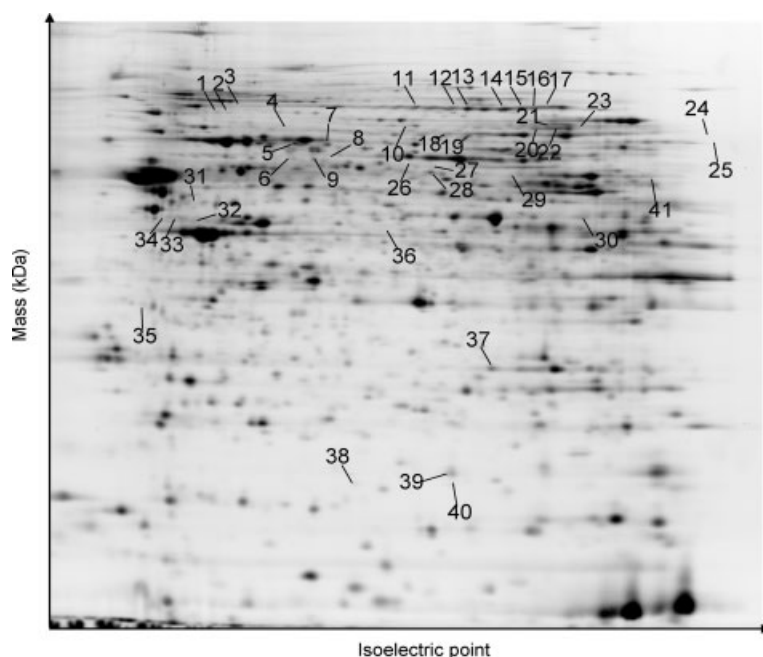
change ( $\geq 1.5$ -fold) between the two groups. Detected differences in protein abundance ranged up to approximately tenfold. Compared to the control samples, 24 spots displayed higher abundance and 29 spots displayed lower abundance in the 10 min postmortem samples. It was possible to match and pick 41 of these spots from the preparative gels for protein identification analysis. Utilizing nano-LC-MS/MS, 28 of the picked spots were successfully identified (Table 2). The identified proteins belong to a number of different protein families including structural proteins (dynamin 1, glial fibrillary acidic protein, cofilin-1, and actin), metabolic proteins (fumarate hydratase 1, pyruvate kinase, and glycyl-tRNA synthetase), and other proteins with specific functions in the brain (gamma-enolase, synapsin 1, and synapsin 2). Changes in abundance were distributed over the entire spot map, regardless of protein abundance, demonstrating that low and high molecular weight proteins, basic and acidic proteins were affected. In some cases, the same protein was identified from several spots demonstrating multiple forms on the same integrity. Evidently, the relative abundance of these forms is dependent on sample preparation. In particular, the protein trail represented by dynamin 1 (spots 12–17) was detected where the most acidic forms were present at higher levels in the control samples.

### 3.2.2 Postmortem time effect on the level of MAPK phosphorylation

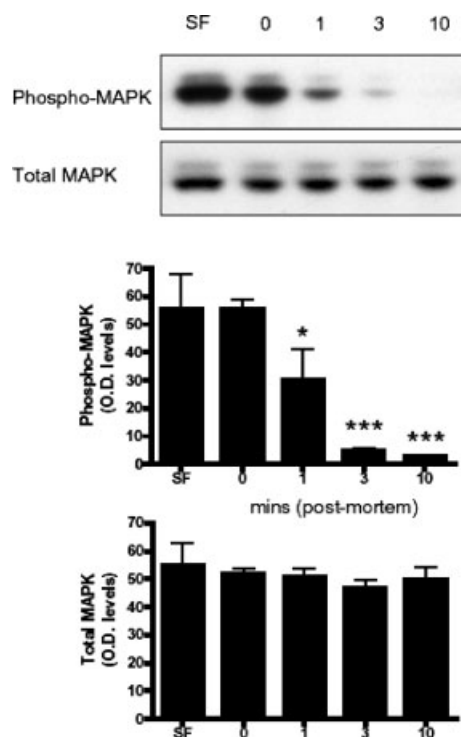
Dually phosphorylated Thr-202/Tyr-205-MAPK was quantified by phospho-specific antibodies using Western blotting in the striatum of the brain. After 10 min postmortem the phosphorylated form of MAPK (Fig. 5) was decreased by  $95 \pm 1\%$  SEM compared to the control group. In contrast, when the MAPK protein was quantified using nonphospho-specific antibodies the levels of the proteins were stable. The total levels of protein MAPK concentration was not significantly altered by postmortem time.



**Figure 3.** Discovery of a peptide biomarker for sample integrity determination. The stathmin fragment 2-20 significantly increases with postmortem time compared to the control group. (a) Mouse hypothalamic tissue 0, 1, 3, and 10 min postmortem delay before sample inactivation ( $***p < 0.001$ , ANOVA,  $t$ -test). (b) Human cingulate cortex ( $n = 12$ ). Six samples (filled boxes) were proteolytically inactivated prior to sample preparation by the Denator instrument and six other samples (open triangles) were prepared at 4°C without inactivation.



**Figure 4.** 2-D DIGE image of mouse cortical proteins. The image represents a spot map generated with internal standard (pooled aliquots of equal amounts of each individual sample). Numbered spots were selected for identification by nano-LC-MS/MS analysis.



**Figure 5.** Postmortem time effect and result of instant tissue fixation of snap-frozen nonmicrowave-irradiated striatal brain tissue on the level of MAPK phosphorylation. The MAPK protein is dually phosphorylated (Thr-202/Tyr-205-MAPK) and is rapidly dephosphorylated with increasing postmortem time (\* $p < 0.05$ , \*\*\* $p < 0.001$ , ANOVA,  $t$ -test). Instant fixation of snap-frozen (SF) brain tissue using the novel sample inactivation instrument (Denator) displayed similar results on MAPK phosphorylation compared to the focused microwave-irradiated control group.

### 3.2.3 Effect of instant tissue fixation device and thawing on snap-frozen nonmicrowave-irradiated brain tissue

Using a tissue fixation instrument, *i.e.*, rapid heat denaturing of frozen brain tissue that immediately has been frozen in liquid nitrogen after sacrifice, showed analogous peptide display results compared to the focused microwave-irradiated control group. The identities of the detected peptides using this sample fixation method included, *e.g.*, the classical neuropeptides such as substance P, leu- and met-enkephalin, and neurotensin.

In contrast, a large number of peptides ( $2800 \pm 550$ ) from high abundant proteins could be detected in the snap-frozen tissue that had been thawed and not fixated by the tissue fixation instrument. The identities of the protein fragments were in concordance to the peptides detected 1, 3, and 10 min postmortem in the time study (Table 1 of Supporting Information).

## 4 Discussion

One of the most significant problem in neuroproteomics is involving sample preparation. Postmortem tissues are subjected to protein degradation and generates a different proteome to what is typically present *in vivo*. Postmortem studies of degradation of proteins have been described in many reports but time after death is often counted in hours [18]. Instead of focusing on individual proteins we have in the present report for the first time also studied the protein degradation products.



**Table 2.** Identities of proteins significantly altered when the focused irradiated microwave control group was compared with the 10 min postmortem group

Spot no. <sup>a)</sup>	UniProtKB <sup>b)</sup>	Protein name	MASCOT score <sup>c)</sup>	Number of identified peptides (>30) <sup>d)</sup>	Sequence coverage (%) <sup>e)</sup>	Average ratio <sup>f)</sup>	Significance <sup>g)</sup>
3	P63038	Heat shock protein 60 kDa	1556	14	45	2.15	**
5	O08553	Dihydropyrimidinase-like 2	1974	13	28	−1.9	**
7	O08553	Dihydropyrimidinase-like 2	2062	12	31	−2.0	***
8	Q62188	Dihydropyrimidinase-like 3	1615	8	21	−2.1	*
9	O08553	Dihydropyrimidinase-like 2	737	11	25	1.8	**
10	Q9CZD3	gi 21264024 or q9czd3 Glycyl-tRNA synthetase	1040	14	24	1.9	**
12	P39053	Dynamin 1	2906	22	33	−2.5	**
13	P39053	Dynamin 1	3130	15	25	−2.19	***
14	P39053	Dynamin 1	1642	15	21	1.9	***
15	P39053	Dynamin 1	3444	22	37	3.1	***
16	P39053	Dynamin 1	1310	11	20	3.4	**
17	P39053	Dynamin 1	1453	13	13	2.3	*
18	O08553	Dihydropyrimidinase-like 2	752	11	24	1.6	**
19	O08553	Dihydropyrimidinase-like 2	1368	10	28	2.0	**
23	O88935	Synapsin 1	314	8	26	1.9	*
24	O88935	Synapsin 1	1094	12	34	−4.0	**
28	Q62188/P52480	Dihydropyrimidinase-like 3/pyruvate kinase	1747/893	9/8	20/25	1.6	**
29	P52480/Q64332	Pyruvate kinase/synapsin 2	288/271	8/4	19/13	−1.6	*
30	P97807/P35486	Fumarate hydratase 1/Pyruvate dehydrogenase E1 alpha1	596	10	18	1.9	**
31	P03995/Q9R111	Glial fibrillary acidic protein/Guanine deaminase	800/471	7/6	11/19	2.5	*
32	Q9CZ44	NSFL cofactor P47 protein	802	12	46	−1.5	**
33	P17183	Gamma-enolase	284	6	19	−1.6	**
34	P17183	Gamma-enolase	328	7	25	−1.7	**
35	Q9D8Y0	EF hand domain containing protein 2	415	10	37	−2.1	***
36	P60710	Actin cytoplasmic 1	502	7	28	−2.2	**
37	Q9DBJ1	Phosphoglycerate mutase 1	126	4	18	−1.5	*
39	P18760	Cofilin-1	411	7	46	−10.9	***
41	Q64332	Synapsin 2	237	4	11	1.7	*

The proteins were separated and detected utilizing DIGE and identified with nano-LC-MS/MS analysis.

a) Spot numbers correlate to the spot numbering on the gel in Fig. 4.

b) Accession number in the UniProtKB.

c) MASCOT score concerning the protein.

d) Number of peptides with a MASCOT score >30 indicating significant identity or extensive homology to sequenced peptides ( $p < 0.05$ ).

e) Sequence coverage calculated from the length and the set of peptides assigned to the protein.

f) Mean value of the abundance ration from the two experimental replicates (see Section 2). A positive value represents higher protein abundance in the 10 min samples and a negative value represents higher protein abundance in the 0 min samples. Notably, similar fold changes were obtained in both experiments indicating consistency between the replicates.

g) Student's *t*-test was performed with control samples and samples 10 min as groups. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.0001$ . Notably, similar *p*-values were obtained in both experiments indicating consistency between the replicates.

Little is known about the course of event the first minute after a sample is removed from its supportive *in vivo* environment. To ensure homeostasis and cell survival, a constant supply of oxygen and nutrients is needed. Without oxygen, oxidative phosphorylation and subsequently adenosine triphosphate (ATP) production is halted, causing deficiencies in cell functions and the cell is

unable to hold the osmotic ion balance due to the energy loss [19]. Organelles within the cell collapse due to the osmotic pressure and cause membranes to disrupt vesicles that contain neuropeptides. The neuropeptides are released and both the neuropeptides and the proteins are rapidly degraded by tissue-specific peptidases and proteases.

The most significant effect in the present study was increased number of peptides detected already within 1 min postmortem in the hypothalamus of the brain. The number of detected peptides continued to increase with increasing postmortem times during the 10 min time-course. After 10 min postmortem the protein fragments were the dominating content of the sample. Many of these fragments originated from high abundant proteins such as hemoglobin, stathmin, cytochrome C oxidase, NADH dehydrogenase, beta actin, and alpha-synuclein.

In some cases the identified neuropeptides were relatively stable over the postmortem time-course, indicating hormone-like functions [20]. Modifications of proteins and peptides, *e.g.*, phosphorylations on the MAPK and the peptide CLIP, were reduced in the striatum already 1 min postmortem, which is in concordance with other studies [21]. The rapid heat transfer inactivation method produced similar results on protein phosphorylations and the number of detected endogenous peptides as the focused microwave irradiation method, indicating a rapid inactivation of proteases. Furthermore, using the gel-based DIGE proteomics approach, it was found that 53 out of approximately 1500 protein spots were significantly changed more than 1.5-fold between control and 10 min postmortem in the brain area cortex. Corresponding peptide fragments from some of these proteins, *e.g.*, dihydropyrimidinase-like protein 2, tubulin  $\alpha$ , and actin were identified among the nonspecifically generated proteolytic peptides. However, the fact that different brain regions were used for the various analytical approaches used limits the findings for these comparisons.

Interestingly, the level of a short N-terminal fragment of the protein stathmin was found to correlate with the general level of degradation and was detected already after 1 min postmortem. Stathmin is ubiquitously expressed and well conserved among many species, including mouse and human, making it a potential marker for sample quality. Previously, proteins, and not peptides, have been used to determine the postmortem status of a sample. As a function of time after death, the postmortem stability of the GABA synthesizing enzyme glutamate decarboxylase (GAD) was studied by using SDS-PAGE and quantitative immunoblotting to measure the rates of degradation of GAD in the cerebral cortex, hippocampus, and cerebellum of rats and mice [22]. The intact 65- and 67 kDa isoforms of GAD (GAD65 and GAD67) disappeared gradually over a 24 h period. In another study the effects of postmortem delay was examined in the mouse brain on glial fibrillary acidic protein, extracellular matrix components, nonphosphorylated neurofilament H, synaptophysin, calbindin, and nitric oxide synthase isoenzymes using histochemical and immunohistochemical methods [23]. A third study used calmodulin binding proteins as indicators of postmortem intervals in rat muscle and lung tissue [24]. Furthermore, Franzen *et al.* [18] detected multiple isoforms of dihydropyrimidinase-related protein-2 in partly degraded mouse brain samples and suggested that the ratio between a truncated form and the full length

protein may be used as an internal control and a biomarker of postmortem time between unrelated samples. However, all of these studies examined relatively long postmortem intervals, the shortest interval being 4 h.

In the present study, several altered proteins postmortem are known to be calpain substrates. Calpain is a family of cysteine proteases that have important roles in the initiation, regulation, and execution of cell death. Dihydropyrimidinase 2 [25], actin [26], dynamin 1 [27], synapsin 1 [28], and enolase [25] are all substrates of calpain. The decrease in oxygen and increase in intracellular  $\text{Ca}^{2+}$  also has the potential to activate  $\text{Ca}^{2+}$  regulated phosphatases and kinases. When the cells are depleted of high energy ATP, the balance is disturbed and the number of phosphorylations is decreased on phosphorylated proteins. This is demonstrated in the present study on MAPK, which lost its phosphorylations on Thr-202/Tyr-205 10 min postmortem. Similar results were obtained when studying phosphorylation on a variety of phosphoproteins in the brain following sacrifice of mice by decapitation, decapitation into liquid nitrogen and focused microwave irradiation [21]. It was found that microwave irradiation generally provided the highest and most consistent levels of protein phosphorylation, regardless of the substrates examined [21]. Furthermore, a striking example of changes in PTMs is demonstrated by the protein trail of dynamin 1 (*cf.* spot 11–17 in Fig. 4). It is likely that the higher levels of the basic forms of dynamin 1 in the 10 min samples represent dephosphorylated forms of the protein. Several functions of dynamin 1 have been reported to be dependent on phosphorylation [29].

The importance of rapid enzyme inactivation in tissue protein analysis was emphasized already in the 1970s when mixtures of nonionic detergents and high concentration of urea, and later thiourea, were used to ensure denaturation [30]. In this report, we present an alternative method for preventing degradation of proteins and peptides in snap-frozen postmortem brain tissue using rapid uniform conductive heat transfer prior to the actual sample preparation procedure. This heating procedure is of advantage for rapid tissue fixation since the proteolytic enzymes are disabled and the tissue can be thawed for a short time, for example when dissecting a sample. This fixation procedure has further advantages, it enables the relatively low-abundant neuropeptides to remain intact, and it minimizes degradation of proteins by proteolysis, and conserves the PTMs of the neuropeptides.

Previous studies focusing on peptides have shown that several peptides are present in higher levels after *in vivo* focused microwave irradiation or after perfusion of the brain with paraformaldehyde than after decapitation [31–33]. However, the use of microwave irradiation techniques is tied to some drawbacks. While some areas of the sample may become overheated and destroyed, others may only partly be inactivated due to inconsistency in the microwave field. In addition, not all types of tissue may be appropriate for focused microwave heating. Other disadvantages are chan-

ges in morphology and the difficulties to use the technique on frozen tissue. The microwave instrument capable of focused irradiation is in addition relatively costly (over \$40 000 for a basic machine) [34]. Our present results show that the alternative method using rapid conductive heat on snap-frozen brain tissue give similar results compared with *in vivo* focused microwave irradiation on proteins, peptides, and PTMs.

The use of rapid conductive heat inactivation does not suffer from the limitations of focused microwave irradiation discussed above. The use of conductive heat gives uniform heating throughout the tissue provides a consistent protein inactivation throughout the whole sample. Conductive heating can be used on fresh as well as frozen tissue with minimal morphological changes enabling post-treatment microdissection. There is no limitation with regards to the tissue source with rapid conductive heat inactivation. Due to the rapid postsampling degradation the use of conductive heat requires a well-planned and prompt sampling procedure with subsequent samples having a similar interval between sampling and inactivation. Furthermore, with conductive heat inactivation, standard animal sacrificial protocols can be followed prior to inactivation, thus precluding the use of novel potentially controversial methods of sacrifice such as focused microwave irradiation. In addition, the instrument is relatively inexpensive compared to the device using focused microwaves.

In conclusion, the present study demonstrates that rapid neuropeptide and protein postmortem changes occur within minutes in brain tissue. Already 1 min postmortem several high abundant proteins are enzymatically degraded resulting in a different composition of the peptidome compared to what is normally present *in vivo*. These protein fragments create a large background of peptides interfering with the neuropeptide detection. A peptide *in situ* marker, stathmin 2-20, was identified for determination of the integrity of the biological sample. An alternative method was presented for protein denaturation and enzyme inactivation for instant fixation of fresh or frozen tissue prior to the sample preparation procedure.

*Birger Scholz at the Department of Pharmaceutical Biosciences, Uppsala University is gratefully acknowledged for valuable discussions; Jesper Hedberg and Sara Sjöberg, GE Healthcare, Uppsala for skillful DIGE analysis; Denator AB for providing the rapid conductive heat inactivation instrument; and the the Stanley Foundation Brain Collection for providing human brain tissue samples. This study was sponsored by the Swedish Research Council (VR) grant no. 11565, 2004-3417, the Swedish Foundation for International Cooperation in Research and Higher Education (STINT) Institutional grant, the K&A Wallenberg Foundation, and the Swedish Knowledge Foundation through the Industrial Ph. D. program in Medical Bioinformatics at the Centre for Medical Innovations (CMI) at the Karolinska Institute.*

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