



# Proteomic analysis of endogenous nitrotryptophan-containing proteins in rat hippocampus and cerebellum

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## Synopsis

Nitration of tryptophan residues is a novel post-translational modification. In the present study, we examined whether NO<sub>2</sub>Trp (nitrotryptophan)-containing proteins are produced in the hippocampus and cerebellum of the adult rat under physiological conditions *in vivo*. Using Western blot analysis with anti-6-NO<sub>2</sub>Trp-specific antibody, we found many similar immunoreactive spots in the protein extracts from both regions. These spots were subsequently subjected to trypsin digestion and LC-ESI-MS/MS (LC-electrospray ionization-tandem MS) analysis. We identified several cytoskeletal proteins and glycolytic enzymes as NO<sub>2</sub>Trp-containing proteins and determined the position of nitrated tryptophan residues with significant ion score levels ( $P < 0.05$ ) in several proteins in both regions. We also observed that the total amount of NO<sub>2</sub>Trp-containing proteins in the cerebellum was significantly greater than that in the hippocampus ( $P < 0.05$ ). Moreover, IP (immunoprecipitation) assays using anti-aldolase C antibody showed that the relative intensity of immunostaining for NO<sub>2</sub>Trp over aldolase C was much higher in cerebellum than in hippocampus. The amounts of nNOS (neuronal nitric oxide synthase) and eNOS (endothelial nitric oxide synthase) were much greater in cerebellum than in hippocampus. This is the first evidence of several specific sites of nitrated tryptophan in proteins under physiological conditions *in vivo*.

**Key words:** cerebellum, hippocampus, 6-nitrotryptophan, proteome, tandem MS analysis, tryptophan residue

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## INTRODUCTION

Protein nitration is a post-translational modification that is induced by RNS (reactive nitrogen species) such as peroxy-nitrite (ONOO<sup>-</sup>) and nitrogen dioxide (NO<sub>2</sub>). ONOO<sup>-</sup> is produced by the very fast reaction between NO and superoxide radical *in vivo*. NO<sub>2</sub> is formed by the reaction between H<sub>2</sub>O<sub>2</sub> and nitrite, which is catalysed with myeloperoxidase and also by decomposition of ONOO<sup>-</sup>. These RNS are known to have the potential to cause oxidation or nitration of various biomolecules. One of the modifications by RNS is the nitration

of tyrosine residues in proteins [1–3]. It has been reported that nitrotyrosine-containing proteins were observed in neurons of AD (Alzheimer's disease) [4,5], Lewy bodies of Parkinson's disease [6] and aged rat cerebellum [7]. Therefore nitrotyrosine has been used as a marker of oxidative and nitrative stress in brain. On the other hand, nitrotyrosine-containing proteins were detected endogenously in the brain of adult mice (9 weeks old) by using proteomic analysis [8]. Moreover, it has been shown that protein tyrosine nitration may regulate cell differentiation [9] and control microtubule dynamics [10]. Thus, protein tyrosine nitration also occurs under physiological conditions *in vivo*.

**Abbreviations used:** AD, Alzheimer's disease; ALP, alkaline phosphatase; DTT, dithiothreitol; eNOS, endothelial nitric oxide synthase; IEF, isoelectric focusing; iNOS, inducible nitric oxide synthase; IP, immunoprecipitation; IPG, immobilized pH gradient; LC-ESI-MS/MS, LC-electrospray ionization-tandem MS; nNOS, neuronal nitric oxide synthase; NO<sub>2</sub>Trp, nitrotryptophan; PDCE2, dihydrolipoly(lysine) residue acetyltransferase component of pyruvate dehydrogenase complex; RNS, reactive nitrogen species; SCOT, succinyl-CoA 3-ketoacid coenzyme A transferase; TBST, Tris-buffered saline with Tween 20

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We have found another type of protein nitration as a novel post-translational modification, namely, formation of NO<sub>2</sub>Trp (nitrotryptophan) in proteins. Although several types of nitrated tryptophan, such as 5- and 6-NO<sub>2</sub>Trp, have been detected through *in vitro* and *in vivo* studies [11,12], we have demonstrated that 6-NO<sub>2</sub>Trp is the major nitrated products [12,13]. Previously, Ishii et al. [14] demonstrated the presence of 27–33 nmol of 6-NO<sub>2</sub>Trp per mol of tryptophan in protease-digested liver of mice after acetaminophen administration by using a quantitative method with LC-ESI-MS/MS (LC-electrospray ionization-tandem MS). Therefore we have developed monoclonal and polyclonal anti-6-NO<sub>2</sub>Trp-specific antibodies and constructed a method to detect 6-NO<sub>2</sub>Trp residue-containing proteins in ONOO<sup>-</sup> modified PC12 cells or their cell lysates by using these antibodies and LC-ESI-MS/MS analyses [15,16]. We identified several glycolytic enzymes and functional proteins as 6-NO<sub>2</sub>Trp residue-containing proteins and determined the positions of 6-NO<sub>2</sub>Trp in the amino acid sequences [16]. In addition, we found changes of 6-NO<sub>2</sub>Trp-containing proteins in the differentiation process from PC12 cells to neuron-like cells induced by NGF (nerve growth factor). We also successfully identified several specific sites of nitrated tryptophan in these proteins [17]. These findings raise the possibility that 6-NO<sub>2</sub>Trp-containing proteins could be detected in physiological conditions *in vivo*. We have chosen rat cerebellum and hippocampus as targets for the formation of 6-NO<sub>2</sub>Trp-containing proteins in physiological processes. Cerebellum contains all three isoforms of NOS (nitric oxide synthase) and the highest levels of nNOS (neuronal NOS), which generate NO as a component of ONOO<sup>-</sup>, compared with other brain regions [18]. Hippocampus CA1 and cerebellar granule cells are known to be more sensitive to oxidative stress than other brain regions under basal conditions [19].

In the present study, we found 12 NO<sub>2</sub>Trp-containing proteins from hippocampus and seven proteins from cerebellum and determined the positions of the nitrated tryptophan residues in these amino acid sequences. We also observed that the total amount of 6-NO<sub>2</sub>Trp-containing proteins in the cerebellum was significantly greater than that in hippocampus ( $P < 0.05$ ). Moreover, we suggested that an increase in nitration rate of the proteins might contribute to the abundance of 6-NO<sub>2</sub>Trp-containing proteins in cerebellum, at least partially, by focusing on aldolase C as a typical example. This is the first evidence of several specific sites of nitrated tryptophan in proteins under physiological conditions *in vivo*.

## MATERIALS AND METHODS

### Animals

The institutional ethics review committee of Juntendo University approved all experimental protocols. Adult male Fischer 344/N rats (6 months of age,  $n = 5$ ) were obtained from Japan SLC Inc. The rats were housed under a 12 h light/12 h dark in an

environmentally controlled room ( $23 \pm 1^\circ\text{C}$  room temperature,  $55 \pm 5\%$  relative humidity) and were provided with food and water *ad libitum*.

### Sample preparation

The rats were deeply anaesthetized with sodium pentobarbital and their brains were removed. Hippocampus and cerebellum were dissected from the brain and frozen in isopentane cooled to approximately  $-80^\circ\text{C}$ . The frozen hippocampus and cerebellum were stored at  $-80^\circ\text{C}$  until use. The frozen hippocampus and cerebellum were homogenized in a lysis buffer containing 40 mM Tris/HCl, 8 M urea, 4% CHAPS, 65 mM DTT (dithiothreitol), 1 mM EDTA, and Complete protease inhibitor (Roche). The homogenates were then centrifuged at 15 000 *g* for 15 min, and the middle layer, containing the proteins, was carefully withdrawn. Protein concentrations were determined by the Bradford method (Protein assay kit; Bio-Rad).

### Two-dimensional electrophoresis and SDS/PAGE

Three samples from each hippocampus and cerebellum were used for proteomic analysis. A portion [200  $\mu\text{g}$  (Western blotting) and 300  $\mu\text{g}$  (Sypro Ruby staining)] of protein was dissolved in the rehydration buffer containing 8 M urea, 4% CHAPS, 40 mM DTT, 0.5% IPG (immobilized pH gradient) buffer and Bromophenol Blue. Then, this solution was applied to an IPG strip (Immobiline<sup>TM</sup> DryStrip pH 3–10 NL, 7 cm; GE Healthcare) and the IPG strip was rehydrated for at least 10 h at  $20^\circ\text{C}$ . Next, IEF (isoelectric focusing) was performed at  $20^\circ\text{C}$  on an Ettan IPG-phor 3 apparatus (GE Healthcare) using a protocol provided by the supplier. After IEF, IPG strips were equilibrated for 15 min in a solution containing 50 mM Tris/HCl (pH 6.8), 6 M urea, 30% glycerol, 2% SDS, 1% DTT and Bromophenol Blue. Two-dimensional SDS/PAGE was carried out using 10% polyacrylamide gels. Subsequently, Western blotting and Sypro Ruby staining were performed. For semi-quantitative analyses of the proteins and enzymes, SDS/PAGE and Western blotting were carried out.

### Western blot analysis

After two-dimensional electrophoresis and standard SDS/PAGE, proteins in gels were transferred on to a PVDF membrane (Immobilon-P, 0.45  $\mu\text{m}$ ; Millipore). Non-specific binding sites were blocked for 1 h at room temperature with Block Ace (DS Pharma Biomedical) and an additional 1 h with non-fat dried skimmed milk powder in TBST (Tris-buffered saline with Tween 20), pH 7.6. The membranes were incubated overnight at  $4^\circ\text{C}$  with primary antibodies followed by 1 h incubation at room temperature with ALP (alkaline phosphatase)-conjugated secondary antibodies. The signals were visualized by chemiluminescence using Immunarstar-AP substrate (Bio-Rad). The primary antibodies used in the present study were as follows: rabbit and mouse anti-6-NO<sub>2</sub>Trp antibody developed in our laboratory [15], mouse anti-nNOS, eNOS (endothelial nitric oxide synthase), iNOS (inducible NOS) antibodies (BD Biosciences), goat

anti-aldolase C antibody (Santa Cruz Biotechnology), and mouse anti- $\beta$ -actin antibody (Sigma–Aldrich). The secondary antibodies used in the present study were as follows: ALP-conjugated anti-mouse IgG Fc-specific (1:50 000, Sigma–Aldrich), ALP-conjugated anti-rabbit IgG Fc-specific (1:50 000, Thermo Scientific–Pierce), ALP-conjugated anti-goat IgG (1:50 000, Millipore Chemicon). For the control experiment for the specificity of the anti-6-NO<sub>2</sub>Trp antibodies, we used a modified method, which was originally reported in a previous study [20] to reduce nitrated proteins. After the transfer, the membranes were exposed to a boiled 0.1 M PBS (pH 7.2) containing 10 mM DTT and 25  $\mu$ M bovine Hb (haemoglobin) (Sigma) or boiled 0.1 M PBS without both DTT and Hb for 3 min. Then, the membranes were washed with TBST, non-specific binding was blocked, and Western blotting was performed. Band densities were determined using Image J software.

### Nano ESI-MS/MS

Sypro Ruby-stained gel spots, which were observed as anti-6-NO<sub>2</sub>Trp-positive signals in the Western blotting, were cut and digested with trypsin. The tryptic peptides were subjected to LC-ESI-MS/MS analysis using a Thermo Fisher Scientific LXQ mass spectrometer with nano-LC (AMR). The LXQ mass spectrometer system consists of a nano-ESI apparatus and an ion trap mass spectrometer. Samples were introduced into the mass spectrometer at 500 nl/min. Typical ESI conditions were as follows: ion spray voltage 1.8 kV, heated capillary temperature 200°C, capillary voltage 40 V and tube lens 115 V. Collision-induced dissociation-MS/MS experiments were performed on mass-selected precursor ions using standard isolation and excitation procedures (activation *q* value 0.25, activation time 30 ms). The collision energy used was 35 (arbitrary units). The conditions of nano-LC were as follows: Magic C<sub>18</sub> column (0.2 mm internal diameter  $\times$  150 mm) and elution with 0.1 % formic acid in 2 % CH<sub>3</sub>CN (solvent A) and 0.1 % formic acid in 90 % CH<sub>3</sub>CN (solvent B) using a programme of 5 % solvent B for 10 min equilibration and a gradient at 2 % solvent B/min for 30 min with a flow rate of 500 nl/min. A database search of Swiss-Prot was performed using the MASCOT search engine (Matrix Science). For identification of 6-NO<sub>2</sub>Trp residues, a modification of 44.99 Da was applied on each of the tryptophan residues. In addition, the following modifications were accounted for during the search: oxidation of Met (+16 Da), methylation of His (+14 Da) and formylation of lysine residue (+28 Da).

### IP (immunoprecipitation)

For IP, 60  $\mu$ l of Protein G–Sepharose 4 Fast Flow beads (GE Healthcare) was washed with IP buffer containing 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 100 mM EDTA, 1 % Nonidet P-40 and Complete protease inhibitor (Roche). The beads were suspended in 1 ml of IP buffer containing 500  $\mu$ g of protein from the homogenates of hippocampus or cerebellum and rotated at 4°C for 1 h to remove proteins which are non-specifically bound to the beads. After rotation, the beads were centrifuged and the supernatants were placed in new microcentrifuge tubes. Subsequently,

new beads and 2  $\mu$ g of goat anti-aldolase C antibody were added to the tubes, and rotated overnight at 4°C. The protein-bound beads were collected by centrifugation and washed three times with IP buffer. The bound proteins were solubilized with sample buffer and incubated at room temperature for 2 h. After incubation, the protein samples were loaded on to 10 % polyacrylamide gels. Then, Western blotting using mouse anti-6-NO<sub>2</sub>Trp and goat anti-aldolase C antibodies was performed.

### Statistical analysis

Results are presented as means  $\pm$  S.D. A paired Student's *t* test was used to analyse the differences between hippocampus and cerebellum. Statistical significance was set at *P* < 0.05.

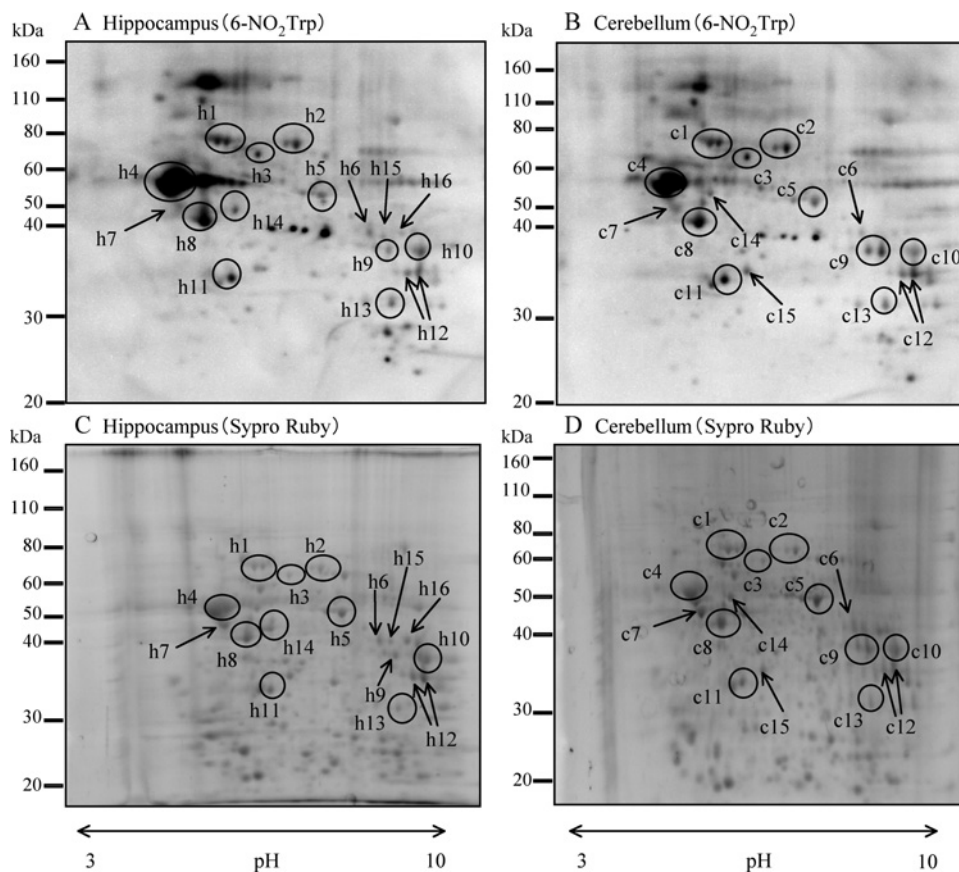
## RESULTS

### Western blotting with the 6-NO<sub>2</sub>Trp antibody

Figure 1 shows representative results of Western blotting with the anti-6-NO<sub>2</sub>Trp antibody (Figures 1A and 1B) and Sypro Ruby staining gels (Figures 1C and 1D) after two-dimensional PAGE of hippocampus (Figures 1A and 1C) and cerebellum (Figures 1B and 1D). Many immunoreactive spots of anti-6-NO<sub>2</sub>Trp antibody were observed in both of the brain regions from the 6-month-old adult rats. Although several immunoreactive spots were detected only in hippocampus or cerebellum, most of the immunoreactive spots were observed in both. In the control experiment, NO<sub>2</sub>Trp was reduced (Figures 2C and 2D) by the method described in the text. The results of the same treatment without reducing agents are shown in Figures 2(A) and 2(B). The immunoreactive spots of rabbit polyclonal and mouse monoclonal anti-6-NO<sub>2</sub>Trp antibody became weak and several spots disappeared by the reduction. Although complete disappearance of the spots had not been attained by this treatment, it has been reported that reduction can be problematic and sometimes may not fully reduce all of the nitrated protein present [21]. Therefore these results indicate the specificity of both anti-6-NO<sub>2</sub>Trp antibodies is considerably high.

### LC-ESI-MS/MS analysis of 6-NO<sub>2</sub>Trp-antibody positive spots

A total of 16 immunoreactive spots and 15 spots on the membrane for the samples from hippocampus and cerebellum respectively were cut out from Sypro Ruby-stained gels and digested with trypsin. The digested samples were analysed with LC-ESI-MS/MS. Putative 6-NO<sub>2</sub>Trp-containing proteins from both regions are shown in Table 1. Although four proteins including mitochondrial electron transfer flavoprotein subunit  $\alpha$  and creatine kinases were identified only in hippocampus and three proteins including voltage-dependent anion-selective channel protein 1 were identified only in cerebellum as putative 6-NO<sub>2</sub>Trp-containing proteins (Table 1), most of the other spots included the same 12 proteins, such as  $\alpha$ -enolase, tubulins and fructose-bisphosphate aldolases (Table 1). We successfully



**Figure 1** Representative results of Western blotting with an anti-6-NO<sub>2</sub>Trp antibody and Sypro Ruby-stained gel after two-dimensional electrophoresis (A) and (C) are Western blotting and Sypro Ruby gel staining of hippocampus respectively. (B) and (D) are those of cerebellum. Open circled spots and spots indicated by arrows in hippocampus (h1–h16) and cerebellum (c1–c15) were subjected to LC-ESI-MS/MS analysis.

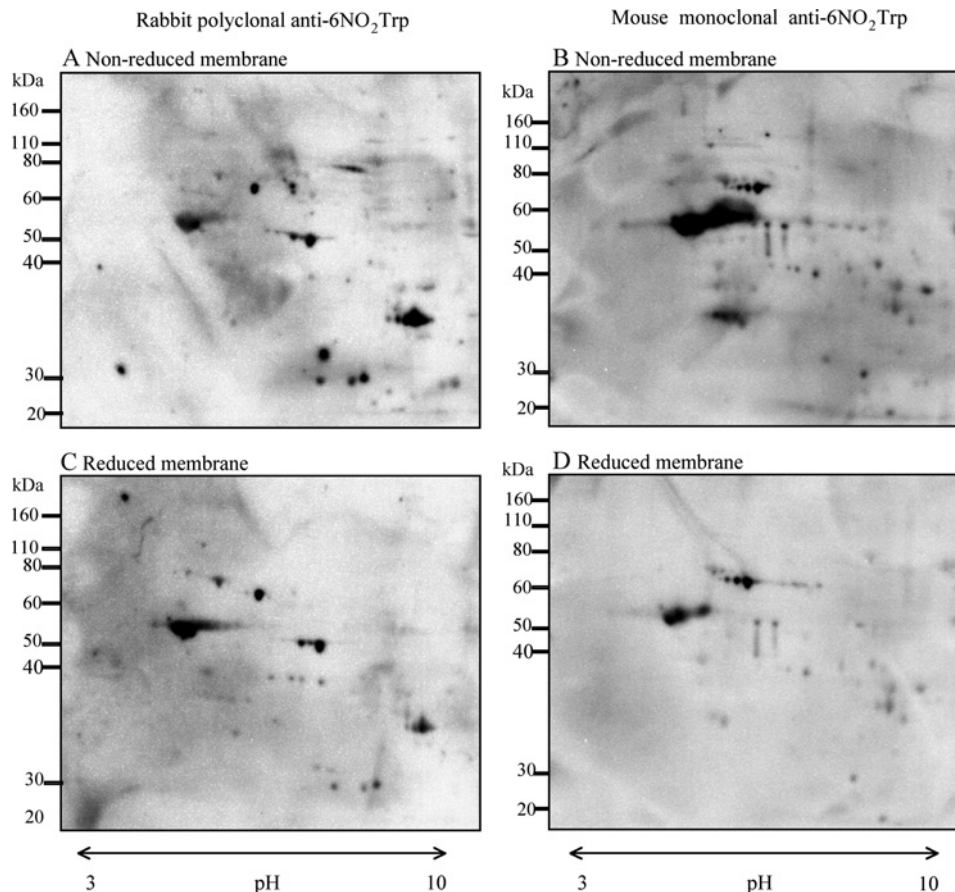
determined the positions of the nitrated tryptophan residues in amino acid sequences of 12 proteins from hippocampus (Table 2) and seven proteins from cerebellum (Table 3). These proteins in both regions included cytoskeletal proteins and glycolytic enzymes. Figure 3 shows the MS/MS spectrum of fructose-bisphosphate aldolase C as a typical example of the MS/MS analyses.

### Semi-quantitative studies of 6-NO<sub>2</sub>Trp-containing enzyme and NOS by Western blotting

In order to compare gross amounts of 6-NO<sub>2</sub>Trp-containing proteins in hippocampus and cerebellum quantitatively, we measured total intensity of the bands on the membrane by Western blot analysis using  $\beta$ -actin as a loading control. Figure 4(A) shows the difference in 6-NO<sub>2</sub>Trp-containing protein bands between hippocampus and cerebellum. The total intensity of 6-NO<sub>2</sub>Trp-containing proteins in the cerebellum was significantly greater than that in hippocampus ( $P < 0.01$ , Figure 4B). We observed the appearance of several new bands and the increased intensity of several bands for the cerebellum (Figure 4A). In order to cla-

rify whether there is a protein with a higher rate of nitration in the cerebellum, we chose spots c9 and h9, which have been identified as aldolase C, as a target protein (Table 1), as the intensity of spot c9 in the Western blotting of two-dimensional electrophoresis was also clearly higher than that of h9 (Figures 1A and 1B). We isolated aldolase C by IP using anti-aldolase C antibody from the extracts of cerebellum and hippocampus and applied the isolated samples to SDS/PAGE and Western blotting. The relative intensity of immunostaining for 6-NO<sub>2</sub>Trp over aldolase C was much higher in cerebellum than in hippocampus (Figure 5). Therefore the increase in the nitration rate of the proteins, as shown in aldolase C, contribute, at least in part, to the increase in gross amounts of 6-NO<sub>2</sub>Trp-containing proteins shown in Figure 4.

Finally, we compared relative amounts of NOS proteins in the extracts from cerebellum and hippocampus. Figure 6 shows the results of Western blot analysis using anti-nNOS, -eNOS and -iNOS antibodies. The amounts of nNOS and eNOS were significantly larger in cerebellum than in hippocampus. We did not observe any difference in protein expression of iNOS between these regions.



**Figure 2** Representative non-reduced and reduced membranes

The samples in the membranes were treated without reducing agents (**A** and **B**) and with reducing agents (**C** and **D**) as described in the text. Immunoreactive spots in (**A**) and (**C**) are detected using rabbit polyclonal anti-6-NO<sub>2</sub>Trp antibody. Immunoreactive spots in (**B**) and (**D**) are detected using mouse monoclonal anti-6-NO<sub>2</sub>Trp antibody.

## DISCUSSION

We have found nitration of tryptophan residues in cultured cells of a model for neuronal differentiation (PC12 cells) and suggested that tryptophan nitration may play a role in the cell-differentiation process in a previous study [17]. In the present study, we focused on rat brain as a target for *in vivo* study. This is the first study to identify several specific sites of nitrated tryptophan on proteins *in vivo* in a physiological state (Tables 2 and 3). Ishii et al. [14] reported the presence of 4- and 6-NO<sub>2</sub>Trp in the protease-digested liver homogenate from mice after administration of acetaminophen by using the LC-ESI-MS/MS method. They found 2.24–3.92 and 26.96–32.71 nmol/mol tryptophan for 4- and 6-NO<sub>2</sub>Trp respectively in the homogenate [14]. This evidence supports *in vivo* formation of NO<sub>2</sub>Trp, although it is from the oxidative stress conditions. On the other hand, formation of 5-hydroxy-6-NO<sub>2</sub>Trp by SCOT (succinyl-CoA 3-ketoacid coenzyme A transferase) has been reported in the heart [22] and kidney [23] of rats. We also found several peptides having an increase in mass of 61 (molecular mass corresponding

to +NO<sub>2</sub>-H + OH-H), which may correspond to the formation of 5-hydroxy-6-NO<sub>2</sub>Trp, with a significant ion score in our system in the present study (results not shown). However, Wang et al. [24] did not find formation of 5-hydroxy-6-NO<sub>2</sub>Trp but found only nitrotyrosine in SCOT from a diabetic model mouse. Therefore formation of 5-hydroxy-6-NO<sub>2</sub>Trp might depend on the biological conditions.

We have shown that the nitration of single tryptophan at position 32 in human recombinant Cu, Zn-SOD (copper/zinc superoxide dismutase) decreases the enzymatic activity [13,25] and the nitration of three tryptophan residues in egg-white lysozyme resulted in total loss of the activity in our previous study [26]. These observations indicate that the nitration of tryptophan residues in enzymes could affect the activity of enzymes. We have investigated the position of the modified tryptophan residues in the three-dimensional structure of the identified proteins in the present study. We found that five of the modified tryptophan residues are located near substrates or coenzyme-binding sites of the enzymes, namely, Trp<sup>616</sup> and Trp<sup>483</sup> in PDCE2 [dihydropoly(lysine)-residue acetyltransferase component of PDH (pyruvate dehydrogenase)

**Table 1 Putative NO<sub>2</sub>Trp-containing proteins in the hippocampus and cerebellum**

All proteins cleared  $P < 0.05$  of the individual ion scores by the Mascot search. Accession numbers are from the Swiss-Prot database. Sequence coverage indicates an average of the samples in each region. 'h' and 'c' under sequence coverage indicate 'hippocampus' and 'cerebellum' respectively.

| Spot number | Protein   | Nominal mass (Da) | Calculated pI value | Sequence coverage (%) |    | Accession number |
|-------------|---|-------------------|---------------------|-----------------------|----|------------------|
|             |   |                   |                     | h                     | c  |                  |
| h1, c1      | Heat-shock cognate 71 kDa protein                                   | 71 055            | 5.37                | 66                    | 76 | P63018           |
| h2, c2      | Serum albumin   | 70 682            | 6.09                | 39                    | 64 | P02770           |
| h3, c3      | PDCE2, mitochondria   | 67 637            | 8.76                | 35                    | 55 | P08461           |
| h4, c4      | Tubulin $\beta$ -2A chain   | 50 274            | 4.78                | 57                    | 77 | P85108           |
|             | Tubulin $\beta$ -2B chain   | 50 377            | 4.78                | 57                    | 77 | Q3KRE8           |
|             | Tubulin $\beta$ -2C chain   | 50 225            | 4.79                | 54                    | 69 | Q6P9T8           |
|             | Tubulin $\beta$ -5 chain  | 50 095            | 4.78                | 53                    | 75 | P69897           |
| h5, c5      | $\alpha$ -Enolase   | 47 440            | 6.61                | 69                    | 83 | P04764           |
| h6, c6      | Glutamine synthetase  | 42 982            | 6.64                | 37                    | 61 | P09606           |
| h7, c7      | $\gamma$ -Enolase   | 47 510            | 5.03                | 83                    | 92 | P07323           |
| h8, c8      | Actin, cytoplasmic 2  | 42 381            | 5.23                | 69                    | 86 | P63259           |
| h9, c9      | Fructose-bisphosphate aldolase C                                    | 39 658            | 6.67                | 87                    | 89 | P09117           |
| h10, c10    | Fructose-bisphosphate aldolase A                                    | 39 783            | 8.31                | 70                    | 91 | P05065           |
| h11, c11    | Guanine nucleotide-binding protein G(l)/G(s)/G(t) subunit $\beta$ 1 | 38 151            | 5.6                 | 49                    | 69 | P54311           |
| h12, c12    | GAPDH   | 36 090            | 8.14                | 58                    | 72 | P04797           |
| h13         | Electron transfer flavoprotein subunit $\alpha$ , mitochondria      | 35 272            | 8.62                | 44                    | –  | P13803           |
| h14         | Creatine kinase B-type  | 42 983            | 5.56                | 55                    | –  | P07335           |
| h15         | Phosphoglycerate kinase 1   | 44 909            | 8.02                | 67                    | –  | P16617           |
| h16         | Creatine kinase U-type, mitochondrial                               | 47 398            | 8.02                | 47                    | –  | P25809           |
| c13         | Voltage-dependent anion-selective channel protein 1                 | 30 851            | 8.62                | –                     | 51 | Q9Z2L0           |
| c14         | Glial fibrillary acidic protein                                     | 49 984            | 5.35                | –                     | 94 | P47819           |
| c15         | L-Lactate dehydrogenase B chain                                     | 36 874            | 5.70                | –                     | 64 | P42123           |

complex] in mitochondria, Trp<sup>314</sup> in fructose-bisphosphate aldolase C (Supplementary Figure S1 at <http://www.bioscirep.org/bsr/032/bsr0320521add.htm>), Trp<sup>85</sup> and Trp<sup>311</sup> in GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Supplementary Figure S2 at <http://www.bioscirep.org/bsr/032/bsr0320521add.htm>) and Trp<sup>383</sup> in phosphoglycerate kinase 1 (Supplementary Figure S3 at <http://www.bioscirep.org/bsr/032/bsr0320521add.htm>). We show the three-dimensional structure of human PDCE2 in Figure 7 as a typical example. The amino acid sequences of PDCE2 are 93% identical between human and rat. The positions of the nitrated tryptophan residues are shown in black in Figure 7. Trp<sup>616</sup> and Trp<sup>483</sup> are located within the CoA-binding region. The addition of a nitro group to these tryptophan residues may cause steric hindrance and a different state on the indole  $\pi$  electron, consequently these changes may affect CoA binding and modulate the activity of the enzyme. However, further studies using isolated proteins are required to clarify the effects of the nitration of the tryptophan residues for each of the enzymes.

We observed a very similar pattern of immunoreactive spots between hippocampus and cerebellum (Figure 1). However, there

were fewer identified peptide sequences with nitrated tryptophan in cerebellum (Table 3) than in hippocampus (Table 2). This may be attributable to the difference in relative contents of each protein in the total protein between cerebellum and hippocampus. On the other hand, the relative amount of NO<sub>2</sub>Trp-containing proteins in cerebellum was higher than that of hippocampus upon analysis using SDS/PAGE (Figure 4). This apparent discrepancy can be explained in part by the evidence that proteins with a large molecular mass or extremely low or high pI are not taken into isoelectric gels in two-dimensional electrophoresis [27]. Therefore the amount of unloaded NO<sub>2</sub>Trp-containing proteins may be larger in cerebellum than in hippocampus. We showed that the nitration rate of tryptophan residues in aldolase C in cerebellum was significantly higher than that in hippocampus (Figure 5). This evidence may result in an increased degree of nitration of each of the tryptophan residues in aldolase C or in an increased percentage of nitrated-tryptophan residues in aldolase C. Therefore the larger nitration amount of the proteins in cerebellum is attributed to the larger modification rate of the proteins at least partly. This higher level of nitration of the proteins could be attributable to the increased concentration of RNS in cerebellum. In rat brain,

**Table 2 Nitrotryptophan-containing trypsin-digested peptide sequences identified from hippocampus**

Boldface W indicates nitrated tryptophan residue. All peptides cleared  $P < 0.05$  of the ion scores by the Mascot search. Supplementary Figures S4–S16 are available online at <http://www.bioscirep.org/bsr/032/bsr0320521add.htm>.

| Protein   | Peptide sequence  | MS/MS spectrum            |
|---|---|---------------------------|
| PDCEZ, mitochondrial  | K <sup>475</sup> .VPEANSS <b>W</b> <sup>483</sup> MDTVIR.Q <sup>490</sup>             | Supplementary Figure S4   |
|   | R <sup>606</sup> .VVDGAVGAQ <b>W</b> <sup>616</sup> LAEFKK.Y <sup>623</sup>           | Supplementary Figure S5   |
| Tubulin $\beta$ -2A, -2B, -2C and -5 chains                     | R <sup>77</sup> .SGPFGQIFRPDNFVFGQSGAGNN <b>W</b> <sup>101</sup> AK.G <sup>104*</sup> | Supplementary Figure S6   |
| $\alpha$ -Enolase   | K <sup>358</sup> .LAQSN <b>GW</b> <sup>365</sup> GVMVSHR.S <sup>373</sup>             | Supplementary Figure S7   |
| Glutamine synthetase  | K <sup>25</sup> .IQLMY <b>W</b> <sup>32</sup> VDGTGEGLR.C <sup>42</sup>               | Supplementary Figure S8   |
| $\gamma$ -Enolase   | K <sup>358</sup> .LAQEN <b>GW</b> <sup>365</sup> GVMVSHR.S <sup>373</sup>             | Supplementary Figure S9   |
| Actin, cytoplasmic 2†   | K <sup>68</sup> .YPIEHGIVT <b>W</b> <sup>79</sup> DDMEK.I <sup>85</sup>               | Supplementary Figure S10  |
| Fructose-bisphosphate aldolase C                                | R <sup>304</sup> .ALQASALSA <b>W</b> <sup>314</sup> R.G <sup>315</sup>                | Figure 2                  |
| Fructose-bisphosphate aldolase A                                | R <sup>304</sup> .ALQASALKA <b>W</b> <sup>314</sup> GGK.K <sup>318</sup>              | Supplementary Figure S11  |
| GAPDH   | K <sup>84</sup> . <b>W</b> <sup>85</sup> GDAGAEYVVESTGVFTTMEK.A <sup>106</sup>        | Supplementary Figures S12 |
|   | K <sup>307</sup> .LIS <b>W</b> <sup>311</sup> YDNEYGYSNR.V <sup>322</sup>             | Supplementary Figures S13 |
| Electron transfer flavoprotein subunit $\alpha$ , mitochondrial | K <sup>187</sup> .APSSSSAGISE <b>W</b> <sup>199</sup> LDQK.L <sup>204</sup>           | Supplementary Figure S14  |
| Phosphoglycerate kinase 1‡                                      | K <sup>382</sup> . <b>W</b> <sup>383</sup> NTEDKVSHVSTGGGASLELLEGG.V <sup>407</sup>   | Supplementary Figure S15  |
| Creatine kinase U-type, mitochondrial                           | K <sup>257</sup> .SFL <b>W</b> <sup>262</sup> VNEEDHTR.V <sup>271</sup>               | Supplementary Figure S16  |

\*This peptide sequence is identical among four isoforms of tubulin  $\beta$  identified in the present study, and tubulin  $\beta$  was counted as one protein.

†Methylation of histidine residue (+14 Da) is added for the identification of nitrated tryptophan residue.

‡Formylation of lysine residue (+28 Da) is added for the identification of nitrated tryptophan residue.

**Table 3 NO<sub>2</sub>Trp-containing trypsin-digested peptide sequences identified from cerebellum**

Boldface W indicates nitrated tryptophan residue. All peptides cleared  $P < 0.05$  of the individual ion scores by the Mascot search. Supplementary Figures S17–S23 are available online at <http://www.bioscirep.org/bsr/032/bsr0320521add.htm>.

| Protein   | Peptide sequence  | MS/MS spectrum           |
|---|---|--------------------------|
| Tubulin $\beta$ -2A, -2B, -2C and -5 chains         | R <sup>77</sup> .SGPFGQIFRPDNFVFGQSGAGNN <sup>101</sup> <b>W</b> AK.G <sup>104*</sup> | Supplementary Figure S17 |
| $\alpha$ -Enolase                                   | K <sup>358</sup> .LAQSN <b>GW</b> <sup>365</sup> GVMVSHR.S <sup>373</sup>             | Supplementary Figure S18 |
| $\gamma$ -Enolase                                   | K <sup>358</sup> .LAQEN <b>GW</b> <sup>365</sup> GVMVSHR.S <sup>373</sup>             | Supplementary Figure S19 |
| Actin, cytoplasmic 2†                               | K <sup>68</sup> .YPIEHGIVT <b>W</b> <sup>79</sup> DDMEK.I <sup>85</sup>               | Supplementary Figure S20 |
| Fructose-bisphosphate aldolase C                    | R <sup>304</sup> .ALQASALSA <b>W</b> <sup>314</sup> R.G <sup>315</sup>                | Supplementary Figure S21 |
| GAPDH   | K <sup>307</sup> .LIS <b>W</b> <sup>311</sup> YDNEYGYSNR.V <sup>322</sup>             | Supplementary Figure S22 |
| Voltage-dependent anion-selective channel protein 1 | K <sup>61</sup> .YR <b>W</b> <sup>64</sup> TEYGLTFTEK.W <sup>75</sup>                 | Supplementary Figure S23 |

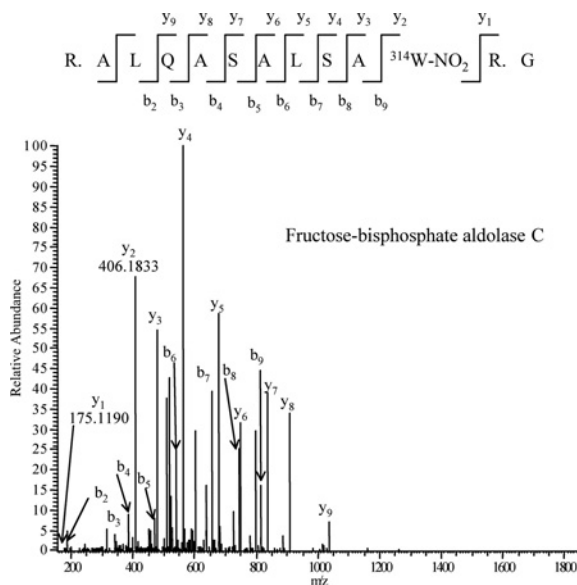
\*This peptide sequence is identical among four isoforms of tubulin  $\beta$  identified in the present study, and tubulin  $\beta$  was counted as one protein.

†Methylation of His (+14 Da) is added for the identification of nitrated tryptophan residue.

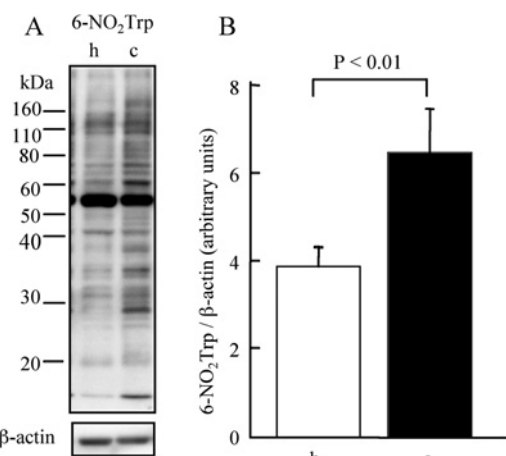
the enzyme activity and expression of nNOS in cerebellum are higher than those in hippocampus [28,29]. In the present study, we have confirmed that cerebellum contains significantly larger amounts of nNOS and eNOS than hippocampus. Thus the expression levels of nNOS and eNOS may affect the difference of NO<sub>2</sub>Trp content between hippocampus and cerebellum. In other words, the expression level of NOS may regulate the nitration of tryptophan residues as a post-translational modification. In normal rat brain, using light and electron microscopy, nitrotyrosine immunoreactivity was shown to be higher in brain regions enriched in nNOS-containing neurons and/or near the neurons, indicating that the expression level of nNOS affects protein nitration [30]. This observation supports the above-mentioned hypothesis.

Finally, we describe the possible significance of the endogenous formation of 6-NO<sub>2</sub>Trp in cerebellum and hippocampus. Formation of 3-nitrotyrosine in proteins by RNS has been widely reported in a number of pathological states associated with in-

flammation [1–3]. We have identified several proteins that contain nitrated tryptophan from affected areas of a model mouse of an inflammatory disease (H. Kawasaki, M. Tominaga, K. Takamori, H. Ogawa, A. Shigenaga and F. Yamakura, unpublished results). This model mouse is known to have oxidative stress in the affected areas. Therefore nitration of tryptophan residues may be caused widely under oxidative stress conditions *in vivo*, which is the same as the case of nitrotyrosine formation. Previously, Sacksteder et al. [8] found 31 unique nitrotyrosine sites within 29 different proteins in a whole mouse brain under basal conditions using LC/LC-MS/MS analyses. They pointed out that more than half of the tyrosine-nitrated proteins are involved in Parkinson's disease or AD. Therefore they suggested that these endogenously nitrated proteins have tyrosine residues specifically sensitive to nitration under mild conditions of oxidative and nitrative stress as a possible concept. Similar to this situation, the first significance for the endogenous formation of NO<sub>2</sub>Trp could be at sites sensitive to mild oxidative and nitrative stress.



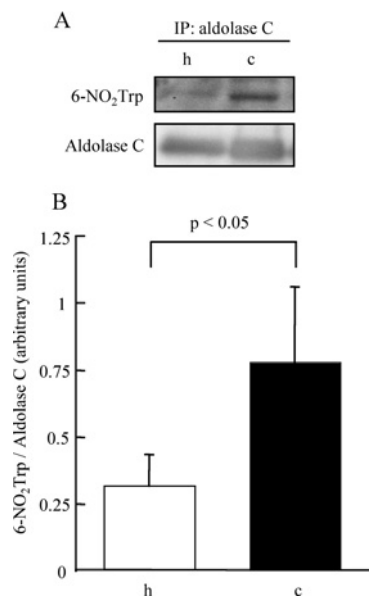
**Figure 3 MS/MS spectrum of the tryptic peptide 304R. ALQASALSA<sup>314</sup>W-NO<sub>2</sub>R. G<sup>315</sup> from fructose-bisphosphate aldolase C**  
W-NO<sub>2</sub> indicates NO<sub>2</sub>Trp residue.



**Figure 4 Semi-quantitative studies of 6-NO<sub>2</sub>Trp-containing proteins**

(A) Representative result of Western blotting with an anti-6-NO<sub>2</sub>Trp antibody of hippocampus and cerebellum. (B) The difference in the relative amounts of NO<sub>2</sub>Trp-containing proteins between hippocampus and cerebellum measured by densitometry. h, hippocampus; c, cerebellum.

Three of the NO<sub>2</sub>Trp-containing enzymes that we identified in the present study (creatine kinase, actin and tubulin  $\beta$ ) are related to AD and Parkinson's diseases [8]. Although we used adult rats (6 months old), not aged rats, they could have a low level of oxidative/nitrative stress in brain under normal conditions. Since NOS levels are much higher in cerebellum than in hippocampus (Figure 6), these stresses may be higher in cerebellum (Figure 4). Nitrotryptophan could be used as a sensitive marker to detect mild oxidative/nitrative stress *in vivo*.

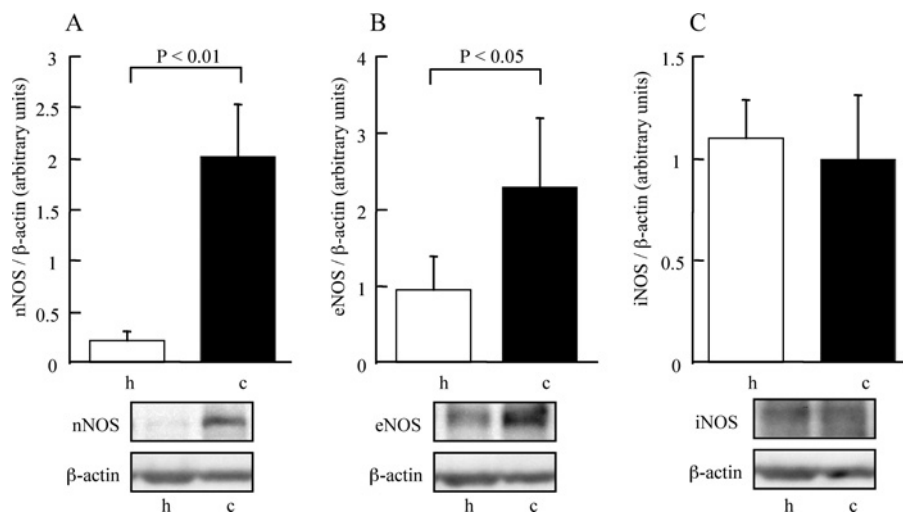


**Figure 5 Comparison of the degree of tryptophan nitration of fructose-bisphosphate aldolase C between the hippocampus and cerebellum**

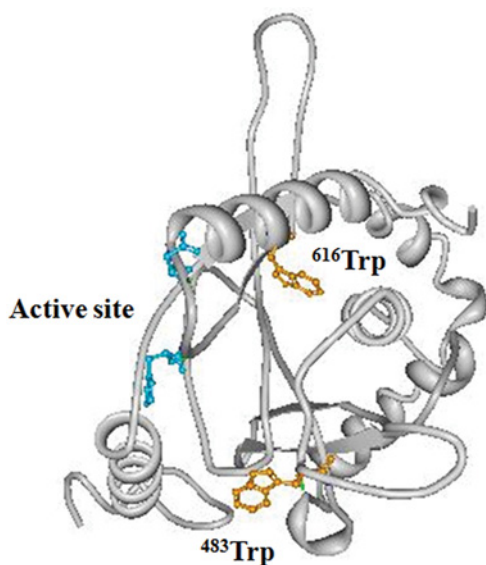
(A) Representative results of Western blotting for anti-6-NO<sub>2</sub>Trp antibody and anti-aldolase C antibody after IP with anti-aldolase C antibody. (B) The difference in the degree of tryptophan nitration between hippocampus and cerebellum measured by densitometry. h, hippocampus; c, cerebellum.

The additional significance of endogenous NO<sub>2</sub>Trp formation is that it could have some physiological functions. The formation of nitrotyrosine has been proposed not only as a marker of oxidative/nitrative damage but also as a functionally relevant post-translational modification of proteins under normal physiological conditions [31]. Three major effects on protein function, that is, no change in protein function, loss of function and gain of function, due to tyrosine nitration can be envisaged. We have found changes in the nitration of tryptophan residues in several proteins during the differentiation process from PC12 cells to neuron-like cells and proposed that tryptophan nitration may contribute somewhat to the differentiation process in the cells [17]. Since we have only limited information on the effect of tryptophan nitration on enzyme function, that is, reducing or abolishing the activity of the isolated enzyme [13,25], we could not clarify the mechanism of the functional change of the enzymes by tryptophan nitration. However, many tryptophan residues in enzymes are known to have specific function in the proteins. For instance, carbohydrates are known to bind preferentially to tryptophan residues in proteins [32] and tryptophan residues exhibit important roles for the binding to lipid bilayer membrane in some enzymes [33]. Nitration of the tryptophan residues may change the interaction of the enzymes with those components. Although further studies are required to clarify the mechanism in detail, the participation of tryptophan nitration in some physiological processes of cerebellum and hippocampus could be of significance. As the nitration of tryptophan residues affects enzyme functions by mechanisms different from the nitration of tyrosine residues, studies on the





**Figure 6 Comparison of the protein amounts of NOS isoforms between the hippocampus and cerebellum** (A) nNOS, (B) eNOS, (C) iNOS. Upper part: the difference in the amounts of the proteins estimated by densitometry. h, hippocampus; c, cerebellum.



**Figure 7 Three-dimensional models of active site region of human PDCE2**

The results for three-dimensional modelling were downloaded from the Protein Data Bank. The accession number of the protein is 3B8K. Both Trp<sup>616</sup> and Trp<sup>483</sup> (shown in yellow), which were nitrated in the present study are located within the CoA-binding region and near the catalytic amino acid residues (shown in blue).

tyrosine nitration alone are insufficient to understand whole RNS functions in physiological processes. Therefore tryptophan nitration is a very important and unique target to be elucidated.

In conclusion, we were able to identify NO<sub>2</sub>Trp-containing cytoskeletal proteins and glycolytic enzymes in both hippocampus and cerebellum. This is the first proteomic study to identify

several NO<sub>2</sub>Trp-containing proteins in physiological conditions *in vivo*. Furthermore, we showed that there is a difference in the amounts of NO<sub>2</sub>Trp-containing proteins between hippocampus and cerebellum, and this difference may result in the difference in the nitration rate of tryptophan residues in the proteins from both regions and the difference in the formation NO in physiological states. These findings suggest the possibility of regulating the function of proteins by the nitration of tryptophan residues in the proteins *in vivo* together with the possibility of a biomarker for oxidative and nitrate stress.

#### AUTHOR CONTRIBUTION

Munehiro Uda designed and performed experiments, analysed the data and wrote the paper. Hiroaki Kawasaki and Takeshi Baba contributed to the discussions and wrote the paper. Ayako Shigenaga contributed to the acquisition and analysing of the LC-MS/MS data. Fumiya Yamakura designed the research and wrote the paper. All authors approved the final paper.

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#### REFERENCES

- 1 Beckman, J. S. and Koppenol, W. H. (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol. Cell Physiol.* **271**, C1424–C1437

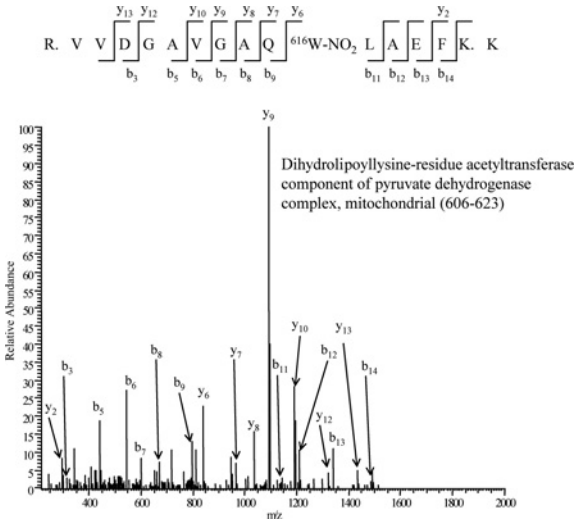


- 2 Greenacre, S. A. and Ischiropoulos, H. (2001) Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. *Free Radical Res.* **34**, 541–581
- 3 Ischiropoulos, H. (2003) Biological selectivity and functional aspects of protein tyrosine nitration. *Biochem. Biophys. Res. Commun.* **305**, 776–783
- 4 Smith, M. A., Richey Harris, P. L., Sayre, L. M., Beckman, J. S. and Perry, G. (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci.* **17**, 2653–2657
- 5 Good, P. F., Werner, P., Hsu, A., Olanow, C. W. and Perl, D. P. (1996) Evidence of neuronal oxidative damage in Alzheimer's disease. *Am. J. Pathol.* **149**, 21–28
- 6 Good, P. F., Hsu, A., Werner, P., Perl, D. P. and Olanow, C. W. (1998) Protein nitration in Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **57**, 338–342
- 7 Gokulrangan, G., Zaidi, A., Michaelis, M. L. and Schoneich, C. (2007) Proteomic analysis of protein nitration in rat cerebellum: effect of biological aging. *J. Neurochem.* **100**, 1494–1504
- 8 Sacksteder, C. A., Qian, W. J., Knyushko, T. V., Wang, H., Chin, M. H., Lacan, G., Melega, W. P., Camp, II, D. G., Smith, R. D., Smith, D. J. et al. (2006) Endogenously nitrated proteins in mouse brain: links to neurodegenerative disease. *Biochemistry* **45**, 8009–8022
- 9 Cappelletti, G., Maggioni, M. G., Tedeschi, G. and Maci, R. (2003) Protein tyrosine nitration is triggered by nerve growth factor during neuronal differentiation of PC12 cells. *Exp. Cell Res.* **288**, 9–20
- 10 Rayala, S. K., Martin, E., Sharina, I. G., Molli, P. R., Wang, X., Jacobson, R., Murad, F. and Kumar, R. (2007) Dynamic interplay between nitration and phosphorylation of tubulin cofactor B in the control of microtubule dynamics. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 19470–19475
- 11 Nuriel, T., Hansler, A. and Gross, S. S. (2011) Protein nitrotryptophan: formation, significance and identification. *J. Proteomics* **74**, 2300–2312
- 12 Yamakura, F. and Ikeda, K. (2006) Modification of tryptophan and tryptophan residues in proteins by reactive nitrogen species. *Nitric Oxide* **14**, 152–161
- 13 Yamakura, F., Matsumoto, T., Ikeda, K., Taka, H., Fujimura, T., Murayama, K., Watanabe, E., Tamaki, M., Imai, T. and Takamori, K. (2005) Nitrated and oxidized products of a single tryptophan residue in human Cu,Zn-superoxide dismutase treated with either peroxynitrite-carbon dioxide or myeloperoxidase-hydrogen peroxide-nitrite. *J. Biochem.* **138**, 57–69
- 14 Ishii, Y., Ogara, A., Katsumata, T., Umemura, T., Nishikawa, A., Iwasaki, Y., Ito, R., Saito, K., Hirose, M. and Nakazawa, H. (2007) Quantification of nitrated tryptophan in proteins and tissues by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **44**, 150–159
- 15 Ikeda, K., Yukihiko Hiraoka, B., Iwai, H., Matsumoto, T., Mineki, R., Taka, H., Takamori, K., Ogawa, H. and Yamakura, F. (2007) Detection of 6-nitrotryptophan in proteins by Western blot analysis and its application for peroxynitrite-treated PC12 cells. *Nitric Oxide* **16**, 18–28
- 16 Kawasaki, H., Ikeda, K., Shigenaga, A., Baba, T., Takamori, K., Ogawa, H. and Yamakura, F. (2011) Mass spectrometric identification of tryptophan nitration sites on proteins in peroxynitrite-treated lysates from PC12 cells. *Free Radical Biol. Med.* **50**, 419–427
- 17 Kawasaki, H., Shigenaga, A., Uda, M., Baba, T., Ogawa, H., Takamori, K. and Yamakura, F. (2011) Nitration of tryptophan in ribosomal proteins is a novel post-translational modification of differentiated and naive PC12 cells. *Nitric Oxide* **25**, 176–182
- 18 Siles, E., Martinez-Lara, E., Canuelo, A., Sanchez, M., Hernandez, R., Lopez-Ramos, J. C., Del Moral, M. L., Esteban, F. J., Blanco, S., Pedrosa, J. A. et al. (2002) Age-related changes of the nitric oxide system in the rat brain. *Brain Res.* **956**, 385–392
- 19 Wang, X., Zaidi, A., Pal, R., Garrett, A. S., Bracer, R., Chen, X. W., Michaelis, M. L. and Michaelis, E. K. (2009) Genomic and biochemical approaches in the discovery of mechanisms for selective neuronal vulnerability to oxidative stress. *BMC Neurosci.* **10**, 12
- 20 Balabanli, B., Kamisaki, Y., Martin, E. and Murad, F. (1999) Requirements for heme and thiols for the nonenzymatic modification of nitrotyrosine. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 13136–13141
- 21 Ye, Y. Z., Strong, M., Huang, Z. Q. and Beckman, J. S. (1996) Antibodies that recognize nitrotyrosine. *Methods Enzymol.* **269**, 201–209
- 22 Rebrin, I., Bregere, C., Kamzalov, S., Gallaher, T. K. and Sohal, R. S. (2007) Nitration of tryptophan 372 in succinyl-CoA:3-ketoacid CoA transferase during aging in rat heart mitochondria. *Biochemistry* **46**, 10130–10144
- 23 Bregere, C., Rebrin, I., Gallaher, T. K. and Sohal, R. S. (2010) Effects of age and calorie restriction on tryptophan nitration, protein content, and activity of succinyl-CoA:3-ketoacid CoA transferase in rat kidney mitochondria. *Free Radical Biol. Med.* **48**, 609–618
- 24 Wang, Y., Peng, F., Tong, W., Sun, H., Xu, N. and Liu, S. (2010) The nitrated proteome in heart mitochondria of the db/db mouse model: characterization of nitrated tyrosine residues in SCOT. *J. Proteome Res.* **9**, 4254–4263
- 25 Yamakura, F., Matsumoto, T., Fujimura, T., Taka, H., Murayama, K., Imai, T. and Uchida, K. (2001) Modification of a single tryptophan residue in human Cu,Zn-superoxide dismutase by peroxynitrite in the presence of bicarbonate. *Biochim. Biophys. Acta* **1548**, 38–46
- 26 Yamakura, F., Ikeda, K., Matsumoto, T., Taka, H. and Kaga, N. (2007) Formation of 6-nitrotryptophan in purified proteins by reactive nitrogen species: a possible new biomarker. *Int. Cong. Ser.* **130**, 22–32
- 27 Becker, M., Schindler, J. and Nothwang, H. G. (2006) Neuroproteomics – the tasks lying ahead. *Electrophoresis* **27**, 2819–2829
- 28 Forstermann, U., Gorsky, L. D., Pollock, J. S., Schmidt, H. H., Heller, M. and Murad, F. (1990) Regional distribution of EDRF/NO-synthesizing enzyme(s) in rat brain. *Biochem. Biophys. Res. Commun.* **168**, 727–732
- 29 Necchi, D., Virgili, M., Monti, B., Contestabile, A. and Scherini, E. (2002) Regional alterations of the NO/NOS system in the aging brain: a biochemical, histochemical and immunochemical study in the rat. *Brain Res.* **933**, 31–41
- 30 Bolan, E. A., Gracy, K. N., Chan, J., Trifiletti, R. R. and Pickel, V. M. (2000) Ultrastructural localization of nitrotyrosine within the caudate-putamen nucleus and the globus pallidus of normal rat brain. *J. Neurosci.* **20**, 4798–4808
- 31 Webster, R. P., Roberts, V. H. and Myatt, L. (2008) Protein nitration in placenta – functional significance. *Placenta* **29**, 985–994
- 32 Malik, A. and Ahmad, S. (2007) Sequence and structural features of carbohydrate binding in proteins and assessment of predictability using a neural network. *BMC Struct. Biol.* **7**, 1
- 33 Ge, C., Georgiev, A., Ohman, A., Wieslander, A. and Kelly, A. A. (2011) Tryptophan residues promote membrane association for a plant lipid glycosyltransferase involved in phosphate stress. *J. Biol. Chem.* **286**, 6669–6684

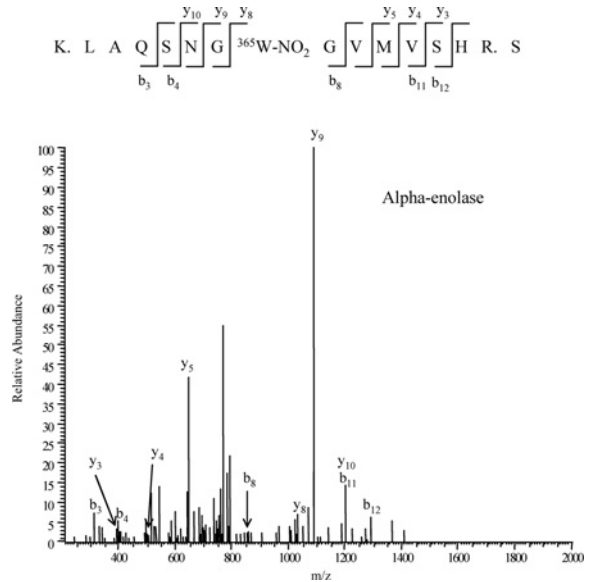
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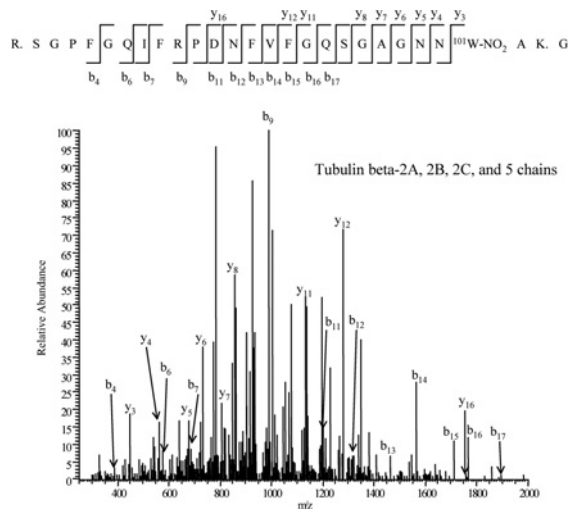




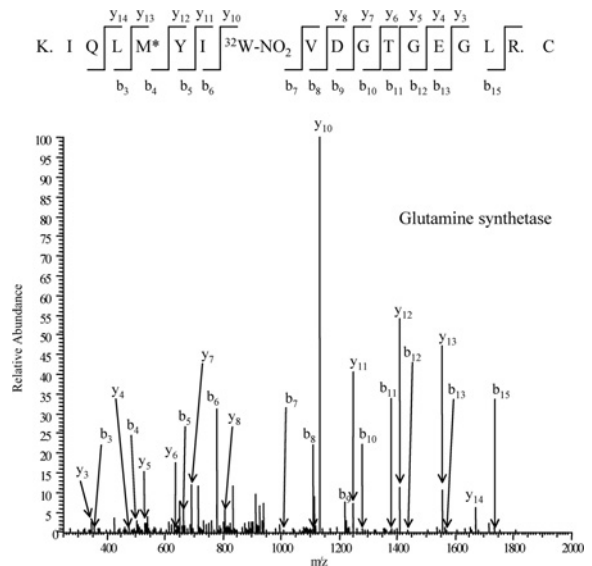
**Figure S5 MS/MS spectrum of the tryptic peptide R<sup>606</sup>.VVDGAVGAQ<sup>616</sup>W-NO<sub>2</sub>LAEFKK.Y<sup>623</sup> from PDEC2, mitochondrial in the hippocampus**  
W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue.



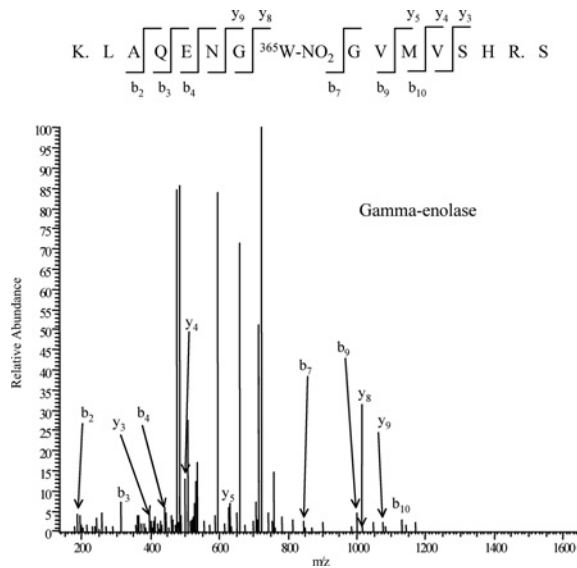
**Figure S7 MS/MS spectrum of the tryptic peptide K<sup>358</sup>.LAQSNQ<sup>365</sup>W-NO<sub>2</sub>GVMVSHR.S<sup>373</sup> from  $\alpha$ -enolase in the hippocampus**  
W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue.



**Figure S6 MS/MS spectrum of the tryptic peptide R<sup>77</sup>.SGPFQIFRPDNFVFGQSGAGNN<sup>101</sup>W-NO<sub>2</sub>AK.G<sup>104</sup> from tubulin  $\beta$ -2A, 2B, 2C and 5 chains in the hippocampus**  
W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue.

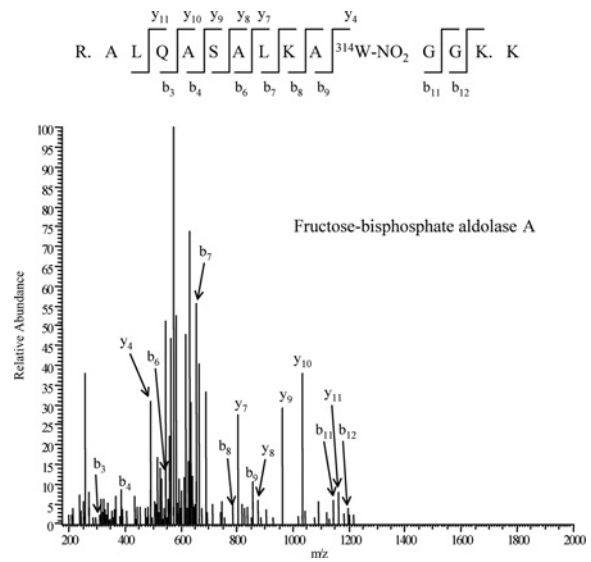


**Figure S8 MS/MS spectrum of the tryptic peptide K<sup>45</sup>.IQLMYI<sup>32</sup>W-NO<sub>2</sub>VDGTGGLR.C<sup>42</sup> from glutamine synthetase in the hippocampus**  
W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue. M\* indicates oxidation of methionine residue.



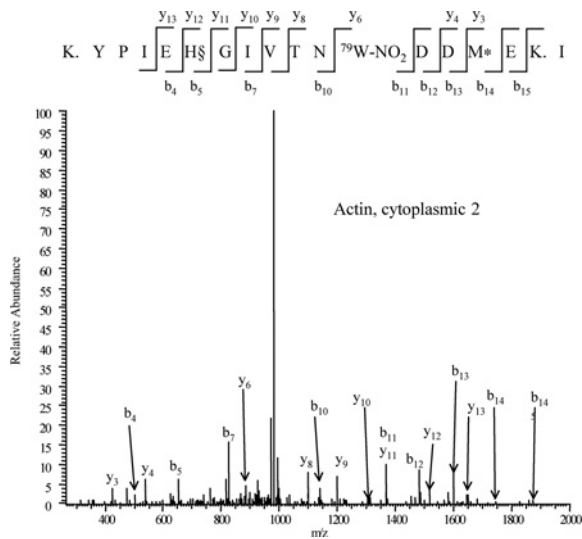
**Figure S9 MS/MS spectrum of the tryptic peptide K<sup>358</sup>.LAQENG<sup>365</sup>W-NO<sub>2</sub>GVMVSHR.S<sup>373</sup> from  $\gamma$ -enolase in the hippocampus**

W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue.



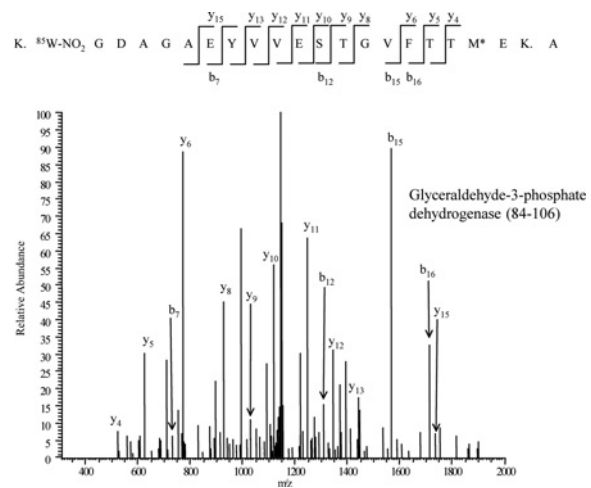
**Figure S11 MS/MS spectrum of the tryptic peptide R<sup>304</sup>.ALQASALKA<sup>314</sup>W-NO<sub>2</sub>GGK.K<sup>318</sup> from fructose-bisphosphate aldolase A in the hippocampus**

W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue.



**Figure S10 MS/MS spectrum of the tryptic peptide K<sup>68</sup>.YPIEHGIVTN<sup>79</sup>W-NO<sub>2</sub>DDMEK.I<sup>85</sup> from actin, cytoplasmic 2 in the hippocampus**

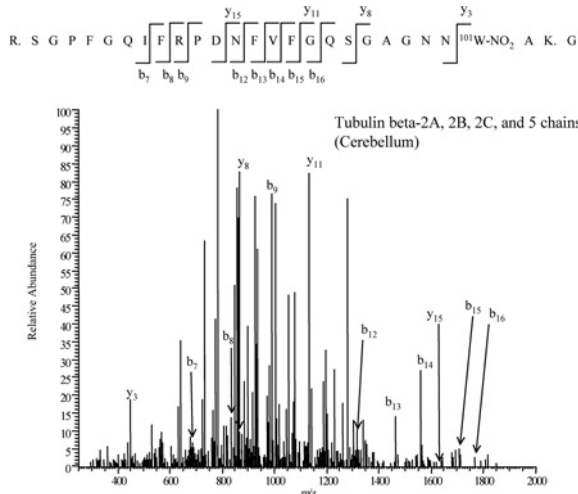
W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue. H $\delta$  indicates the methylation of the histidine residue. M\* indicates oxidation of the methionine residue.



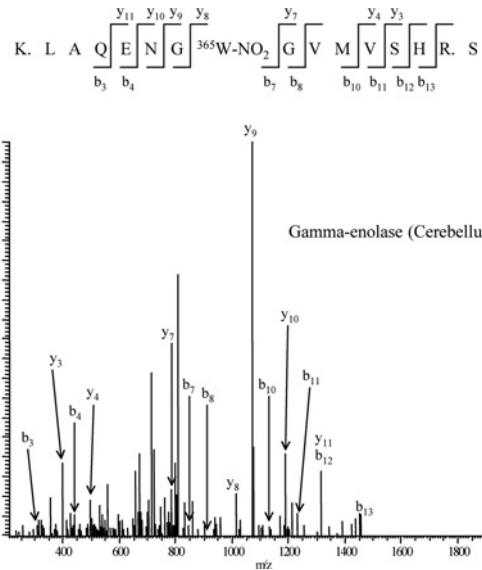
**Figure S12 MS/MS spectrum of the tryptic peptide K<sup>84</sup>.W<sup>85</sup>-NO<sub>2</sub>GDAGAEYVVESTGVFTTMEK.A<sup>106</sup> from GAPDH in the hippocampus**

W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue. M\* indicates oxidation of methionine residue.

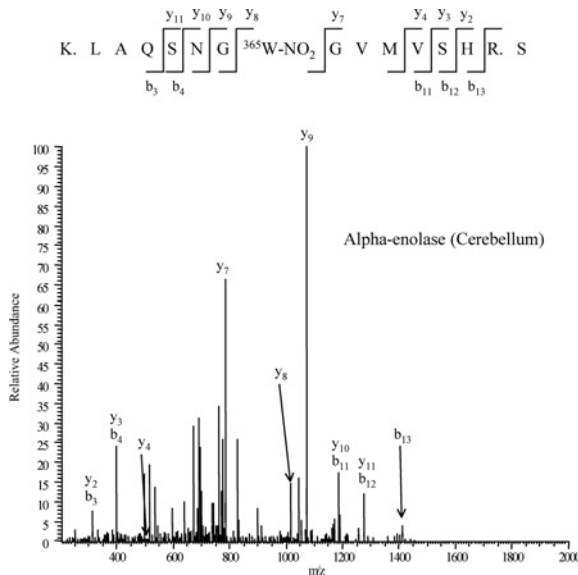




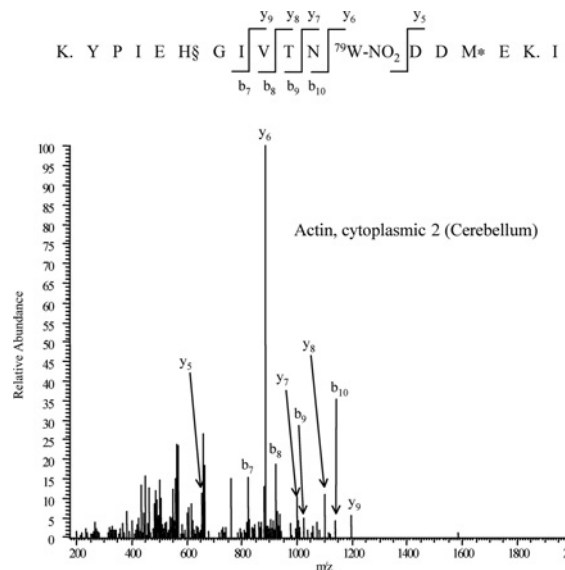
**Figure S17 MS/MS spectrum of the tryptic peptide R<sup>77</sup>.SGPFQIFRPDNFVFGQSGAGNN<sup>101</sup>W-NO<sub>2</sub>AK.G<sup>104</sup> from tubulin β-2A, -2B, -2C and -5 chains in the cerebellum**  
W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue.



**Figure S19 MS/MS spectrum of the tryptic peptide K<sup>358</sup>.LAQENG<sup>365</sup>W-NO<sub>2</sub>GVMVSHR.S<sup>373</sup> from γ-enolase in the cerebellum**  
W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue.



**Figure S18 MS/MS spectrum of the tryptic peptide K<sup>358</sup>.LAQENG<sup>365</sup>W-NO<sub>2</sub>GVMVSHR.S<sup>373</sup> from α-enolase in the cerebellum**  
W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue.



**Figure S20 MS/MS spectrum of the tryptic peptide K<sup>68</sup>.YPIEHGIVTN<sup>79</sup>W-NO<sub>2</sub>DDMEK.I<sup>85</sup> from actin cytoplasmic 2 in the cerebellum**  
W-NO<sub>2</sub> indicates the NO<sub>2</sub>Trp residue. H§ indicates the methylation of the histidine residue. M\* indicates the oxidation of the methionine residue.

