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Expression of Nicotinamide N-Methyltransferase (E.C. 2.1.1.1) in the Parkinsonian Brain

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Abstract. Nicotinamide N-methyltransferase (NNMT) has been proposed as a link between the environmental and genetic factors of Parkinson disease (PD). Therefore, we explored the hypothesis that high levels of NNMT expression may predispose to the development of PD. Regions of high mRNA expression were shown in the spinal cord, medulla, and temporal lobe, with lowest expression in the cerebellum, subthalamic nucleus, and caudate nucleus. Using 2 NNMT antibodies, the protein was shown to be expressed in multipolar neurons in the temporal lobe, caudate nucleus, and spinal cord, granular neurons of the cerebellum, dopaminergic neurons in the substantia nigra, and in the axons of the third nerve. Expression of NNMT was compared in PD and non-PD control cerebella and caudate nucleus. PD tissue exhibited significantly increased levels of NNMT protein and activity. PD disease duration was inversely correlated with the level of expression in cerebellum. This is the first demonstration that patients with PD have higher levels of NNMT activity and protein in brain tissue than those without PD and that NNMT expression is associated with neurons that degenerate in PD.

Key Words: Cerebellum; Methyl-phenyl-tetrahydropyridine (MPTP); Nicotinamide N-methyltransferase (NNMT); Parkinson disease; Pyridine; Substantia nigra.

INTRODUCTION

Nicotinamide N-methyltransferase (NNMT, EC 2.1.1.1) catalyses the N-methylation of nicotinamide (1–3), various closely related structural analogues such as thionicotinamide and 3-acetylpyridine (4), and poor structural analogues such as quinoline, isoquinoline, and 1,2,3,4-tetrahydroisoquinoline to form positively charged pyridinium ions (5–7). Liver NNMT is an S-adenosylmethionine-dependent cytosolic enzyme (1) with a molecular weight of approximately 27 kDa (5, 8). The human NNMT gene has been localized to chromosome 11q23.1, is 16.5 kbp in length, and comprises 3 exons and 2 introns with an open reading frame of 792 base pairs which codes for a protein of 264 amino acids (8, 9). The pharmacogenetics of human liver NNMT have been well described. A large pharmacological study of NNMT activity showed a bimodal frequency distribution with 25% of samples in a high activity subgroup (10). This distribution is due to variations in NNMT protein levels and not as a result of a coding region polymorphism (11).

N-methylation usually results in the detoxification of the substrate. However, paradoxically this biotransformation has been shown to increase the toxicity of some compounds, e.g. the N-methylation of 1,2,3,4-tetrahydroisoquinoline to the dopaminergic neurotoxin N-methyl-1,2,3,4-tetrahydroisoquinoline (4). It has been suggested that the presence of NNMT activity in the brain may be a factor in the etiology of Parkinson disease (PD), as the products of the N-methylation of pyridines by NNMT are related to another well-characterized dopaminergic neurotoxin methylphenylpyridinium ion (12). Recent interest in the potential roles of NAD in cellular signaling pathways suggests a potential role for NNMT in the regulation of various brain cellular pathways (13).

In this work we set out to explore the hypothesis that patients with PD have higher levels of NNMT in the brain than normal subjects. The rationale for this was that higher levels of the enzyme would generate higher concentrations of neurotoxic methylpyridinium compounds. Because the only available tissue was postmortem material from PD patients who have experienced years of illness and because substantial neuronal loss must have occurred in the substantia nigra, if NNMT is expressed in neurons, increased nigral expression in PD would be extremely difficult to detect. Therefore, we investigated 2 regions not greatly affected by the disease (cerebellum and caudate nucleus) and for which high quality tissue was readily available. This enabled expression in control and disease subjects to be compared under circumstances where the direct effect of the disease was minimal. The hypothesis presupposes that NNMT is expressed in neurons and that expression of the enzyme is widespread throughout the brain. In order to establish these points, a series of preliminary studies was carried out on tissue from different individuals. These involved a) determination of regional expression of NNMT mRNA in the normal human central nervous system using Northern blotting, b and c) confirmation of NNMT expression by Western blotting and activity assay, respectively, d) elucidation of cell specific expression by immunohistochemistry, results being compared with those in the liver (positive control tissue) and renal cortex (negative control tissue), and finally e) determination of the stability of

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NNMT in brain tissue. Having determined optimal experimental conditions and confirmed widespread expression of the enzyme in the central nervous system (CNS), a more wide-ranging comparison of enzyme expression in control and parkinsonian brains was carried out. This consisted of comparison of NNMT protein expression (Western blotting) and enzyme activity in 2 regions not greatly affected by PD, cerebellum and caudate nucleus. Secondly, immunohistochemistry was used to compare neuronal expression of NNMT in normal and parkinsonian cerebellum. Finally, a limited immunohistochemical study was carried out to compare neuronal NNMT expression in substantia nigra and oculomotor (third) nerve region in the disease and control subjects.

MATERIALS AND METHODS

All chemicals were of the highest purity available and were obtained from Sigma, Poole, UK, unless otherwise stated. Secondary antibodies and streptavidin peroxidase conjugate used for immunohistochemistry were obtained from The Binding Site Ltd., Birmingham, UK. Goat peroxidase-anti-peroxidase (PAP) was obtained from Dako Ltd., Cambridge, UK. Vecta-Shield mounting medium was obtained from Vector Laboratories (Burlingame, CA). Two anti-NNMT antibodies against linear epitopes of the NNMT protein (NNMT a1 sequence DYSDQNLQELEKWLKKEPEA; NNMT a2 sequence CDLEGNRVKGPEKEEKLRQA) used in the immunochemical investigations described below were produced and affinity-purified as described previously (11, 14). Preabsorption of the NNMT antibodies was carried out using antigenic peptide (Alta Bioscience Ltd., Birmingham, UK) as previously described (14). All studies on regional and nigral NNMT expression used both anti-NNMT antibodies, whereas only antibody 1 was used for the cerebellar studies.

Preliminary Experiments

Regional Expression of NNMT mRNA: Human brain multiple tissue Northern blots were obtained from Clontech (Clontech Laboratories Inc., Basingstoke, UK). NNMT mRNA was detected by probing with a ^{32}P-labelled NNMT cDNA probe prepared as described previously (11), followed by stripping and reprobing with a ^{32}P-labelled ß-actin cDNA probe (Clontech). Densitometric analysis of the bands obtained was performed using a laser densitometer and the ratios of NNMT to ß-actin were calculated to allow inter-regional comparisons to be made.

NNMT Protein Expression Determined by SDS-PAGE/Western Blotting: To confirm the results of the Northern blotting study at the protein level, tissue from 4 brain regions was investigated at the translational level. Normal human temporal lobe was obtained from the Department of Pathology, University of Birmingham, UK, from a 55-yr-old female patient undergoing surgical treatment for epilepsy. It was classed as a pathologically normal tissue because it was taken from an unaffected region and removed only for access. The sample was immediately frozen in liquid nitrogen. Normal human spinal cord was obtained as a postmortem sample from the Department of Pathology, University of Birmingham from a 55-yr-old male patient who had died of a pulmonary embolus and dilated cardiomyopathy. The postmortem interval was 2 days. Normal caudate nucleus and cerebellum were obtained from the Parkinson's Disease Society Brain Research Centre (PDBRC), Institute of Neurology, University College London, UK. All brains used here and in subsequent analyses were subjected to a full neuropathological examination by the PDBRC, who found no evidence of other concomitant disease or pathology. As a confirmatory exercise, expression in an enzyme-positive (liver) and enzyme-negative (renal cortex) was investigated. Normal human liver and kidney were obtained from the Liver Unit of the Queen Elizabeth Hospital, Birmingham, UK as flash-frozen samples taken from a traffic accident victim acting as a donor for juvenile transplants. In this latter case the postmortem interval was less than 24 h.

Homogenates were prepared by homogenizing in 50 mM Tris-buffered saline, pH 7.4 (TBS) and centrifuging the homogenate at 600 $\times$ *g* for 10 min. Protein concentration was determined using the method of Bradford (15). Homogenates (20 μg/well) were subjected to either analysis for NNMT activity as described below or electrophoresed as per the method of Laemmli (16) on a 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subjected to Western blotting as previously described (16), with the modification of using primary antibody dilution of 1:500 overnight at 4°C.

Expression of NNMT Activity: The activity of NNMT of the homogenates described above was determined in triplicate for each sample using a radioisotope assay based upon the method of Rini et al (10). Homogenate was diluted 1:10 (v/v) with bovine serum albumin (BSA, 2 mg/ml) supplemented 5 mM Tris HCl, pH 7.2 and 100 μl was transferred to a 15 ml polypropylene tube. One hundred fifty mM nicotinamide in dimethylsulphoxide or dimethylsulphoxide alone (10 μl) was added and the reaction initiated by the addition of a solution comprising 500 mM Tris HCl, pH 7.2, 150 μM S-adenosylmethionine and S-adenosyl-L-[*methyl*-^{3}H]methionine (12.5 μCi/ml, Amersham, Rainham, UK). The reaction was incubated at 37°C for 20 min and then terminated by the addition of 200 μl of 500-mM borate buffer, pH 10. Eight hundred fifty mM heptanesulphonic acid (100 μl) was added and vortexed for 10 s, after which the radioactive product was extracted into organic phase by the addition of a 3:2 solution of isoamyl alcohol: toluene (5 ml) followed by centrifugation at 400 $\times$ *g* for 10 min. An aliquot (2 ml) of the organic phase was mixed with an equal volume of scintillation fluid (OptiPhase Hisafe 3, LKB Scintillation Products, Leicester, UK) and the radioactivity was counted using a beta counter. Results were corrected for quenching and counting efficiency (62%) and were expressed as units (the formation of 1 nmol of 1-methylnicotinamide per hour per mg protein).

Cellular Localization of NNMT: The cellular localization of NNMT in single samples of normal temporal lobe, spinal cord, normal and parkinsonian caudate nucleus, and 2 normal and parkinsonian cerebella and midbrain sections containing both substantia nigra and third nerve (obtained from the PDBRC) was determined using the method of Sternberger et al (17). In addition, immunohistochemistry was carried out on 2 non-central nervous system tissues; liver, which is known to express the enzyme, and the renal cortex, in which the enzyme activity was undetectable (18). These served as positive and negative control tissues, respectively.

TABLE 1
Optimized Conditions Used for the Immunohistochemistry Procedure Used in this Study

Antibody region	Blocking	NNMT 1°	NNMT 2°	NNMT 3°	THO/GFAP 1°	THO/GFAP 2°	THO/GFAP 3°	Counterstain	Mounting
Temporal lobe	1 h RT	1:50[a]	1:25[c]	1:50[e]	—	—	—	Hematoxylin	DPX
Spinal cord	18 h RT	1:100[a]	1:50[c]	1:100[e]	—	—	—	Hematoxylin	DPX
Cerebellum	1 h RT	1:50[a]	1:25[c]	1:50[e]	1 h RT	1:50[f]	1:50[g]	Hematoxylin	DPX
Substantia nigra/third nerve	1 h RT	1:50[b]	1:50[d]	—	1 h RT	1:50[f]	1:50[g]	Hematoxylin (GFAP/THO only)	VectaShield (NNMT) DPX (GFAP/THO)
Caudate Nucleus	1 h RT	1:50[b]	1:50[d]	—	—	—	—	—	VectaShield
Liver	30 min RT	1:100[a]	1:50[c]	1:100[e]	—	—	—	Hematoxylin	DPX
Kidney	30 min RT	1:100[a]	1:50[c]	1:100[e]	—	—	—	Hematoxylin	DPX

[a] Overnight, 4°C. [b] 1 hour, RT. [c] Donkey-anti-sheep IgG, 1 hour at RT. [d] Donkey-anti-sheep IgG, FITC conjugate, 1 hour at RT. [e] Goat PAP, 1 hour at RT followed by DAB solution. [f] Donkey-anti-sheep IgG, biotin conjugate, 1 hour at RT. [g] Streptavidin, peroxidase conjugate, 1 hour at RT followed by DAB solution. Abbreviations: h, hour; RT, room temperature.

TABLE 2
Patient Data for Spinal Cord Samples

Sex	Age	PMI	Officially recorded cause of death
F	85	34 h	Carcinoma of the breast
M	75	64 h 50 min	Pulmonary embolism
F	85	73 h 30 min	Metastatic carcinoma

Abbreviations: PMI, postmortem interval; h, hour.

Anti-NNMT and other primary antibodies were diluted in 25 mM Tris-buffered saline, pH 7.4 supplemented with 1% (w/v) BSA. Sections of frozen tissue (5 μm) were fixed briefly in ice-cold acetone, demyelinated in chloroform then blocked for peroxidase activity after which the brain tissues only were microwaved at 95°–99°C for 30 min in a 10 mM citrate buffer, pH 6.0. Protein binding sites were blocked using normal donkey serum (1:10, The Binding Site Ltd.) and NNMT expression was detected as described in Table 1. These conditions were optimized in order to produce the highest degree of contrast between staining arising as a result of antibody binding and any endogenous pigmentation that may be present.

The cellular localization of NNMT in both the cerebellum and the substantia nigra was clarified using anti-glial fibrillary acidic protein (GFAP, 1:100, The Binding Site Ltd.) and anti-tyrosine hydroxylase (THO, 1:10, Novo Castra Laboratories Ltd., Newcastle-upon-Tyne, UK) in single-labeling experiments to determine whether the cells expressing NNMT were neuronal or glial cells. Because THO is not expressed in the cerebellum, incubation of this region with anti-THO antibody mainly served as an additional immunohistochemical negative control. Briefly, sections were fixed, blocked for peroxidase activity, microwaved, incubated with donkey serum (1:20), and probed for THO and GFAP expression as described in Table 1.

Stability of NNMT Protein upon Storage: To investigate the stability of the enzyme under storage, 3 snap-frozen spinal cord samples taken from patients who had not died of PD were obtained from the PDBRC as outlined in Table 2. Samples of each spinal cord sample were stored at either 4°C or −20°C for 0, 12, or 24 h prior to homogenization. Samples (n = 3 for each storage condition) were assayed in triplicate for enzyme activity as described above, making a total of 18 assays for each subject sample. Further samples were electrophoresed, blotted to nitrocellulose, and probed with anti-NNMT antibodies as described above. Band detection was done using a combination of donkey-anti-sheep IgG conjugated to horseradish peroxidase (The Binding Site Ltd., 1:1000, 1 h room temperature) followed by electrochemiluminescence (Amersham Pharmacia Biotech) as per manufacturer's instructions. Bands were quantified using laser densitometry (n = 3 per band) and expressed as percentage of zero time.

Quantitation of Expression of NNMT in the Brains of PD and Non-PD Subjects

Flash-frozen normal and parkinsonian cerebellum and caudate nucleus were obtained from the PDBRC as approximately 50-mg pieces of tissue from which protein was isolated for SDS-PAGE/Western blotting and NNMT activity assay as described above. Additionally, normal and parkinsonian cerebella

TABLE 3
Patient Data

Patient ref.	Group	Sex	Age	Officially recorded cause of death	PMI
97.0700	C	F	53	Intra-cerebral hemorrhage	29 h 30 min
97.0701	C	F	77	Chronic obstructive airway disease	79 h 30 min
97.0677	C	M	86	Bronchopneumonia, heart failure	53 h
98.0758	C	M	86	Myocardial infarct	23 h 30 min
94.0485	C	M	43	Heart attack	15 h
94.0446	C	F	73	Unknown	25 h 50 min
95.0585	C	M	73	Bowel Obstruction	<48 h
95.0539	C	M	90	Bronchopneumonia	<48 h
94.0456	C	M	43	Coronary artery occlusion	32 h
94.0443	C	M	77	Myocardial infarction	24 h
92.0291	C	M	56	Ischemic heart disease	4 h
92.0300	C	F	83	Ischemic heart disease	49 h 45 min
93.0373	C	F	80	Myocardial infarct	28 h
94.0482	C	F	88	Cardiac and respiratory arrest	8 h
93.0377	C	M	74	Left ventricular failure	5 h 30 min
94.0457	C	F	93	Bronchitis	9 h
95.0580	PD	F	75	Hemopericardium	28 h 15 min
97.0650	PD	M	84	Chest infection	40 h
97.0681	PD	F	73	Cardiac event	19 h 55 min
96.0642	PD	M	67	PD general deterioration	4 h 17 min
96.0597	PD	F	80	Old age	11 h
97.0661	PD	F	81	Bronchopneumonia	32 h
97.0674	PD	M	71	Bronchopneumonia	38 h
96.0633	PD	F	79	Bronchopneumonia	10 h 45 min
97.0668	PD	F	88	Bronchopneumonia	19 h 45 min

Abbreviations: PMI, postmortem interval; h, hour.

and substantia nigra were obtained as pre-cut 12 μm sections on glass slides for immunohistochemistry. Clinical data on the subjects (postmortem interval, pH of cerebellum, age of death, disease duration [parkinsonian subjects only]) relating to the 25 donors were also obtained; 16 non-PD control and 9 PD as outlined in Table 3. Postmortem intervals were generally below 40 h; however, 1 sample had a postmortem interval of 79 h 30 min. The pH of this brain was within acceptable limits (greater than 6.25), as pH has been shown to be a reliable indicator of agonal state and mRNA preservation (19).

Western Blotting and Enzyme Activity of NNMT in the Caudate Nucleus and Cerebellum

The expression of protein and enzyme activity in the caudate nucleus and cerebellum of all 25 PD and non-PD cases were investigated as described above.

Immunochemical Quantitation of NNMT Expression in Cerebellum and Substantia Nigra

The quantitation of NNMT in the cerebellum of all 25 PD and non-PD cases was investigated using immunohistochemistry as described in Table 1. The granular layer of each section was counted independently by 2 investigators in 20 random areas, counting both immunostained and unstained cells in a standard unit area as visualized using a standard 10 × 10 eyepiece graticule (Pyser-SGI Ltd, Kent, UK). The ratio of stained to total neurons (irrespective of the apparent level of staining) was determined and the results reported as percentage expression.

The quantitation of NNMT in the substantia nigra was determined using immunofluorescence as described in Table 1. Tissue was only available for 7 control and 9 parkinsonian subjects. Neurons in 4 identically sized areas for each subject were counted independently by 2 researchers as for the cerebellum. Some substantia nigra slides were counterstained with propidium iodide (1:10) for 15 min at room temperature prior to mounting.

Statistical Analysis

All statistical analyses carried out using the Instat statistics package (GraphPad, San Diego, CA) were as follows: i) Kruskal-Wallis non-parametric ANOVA and Dunn's Multiple Comparison test was used for assessing the storage properties of tissue; ii) comparisons of data from parkinsonian and control subjects were analyzed using either the Mann-Whitney *U* nonparametric test or the Student *t*-test; and iii) the significance of trends was determined using Spearman rank correlation. Results were considered significant at the 5% level.

RESULTS

Preliminary Experiments

Regional Expression of NNMT mRNA Expression: Table 4 shows the ratio of NNMT:ß actin densities in 16 regions of 1 human brain. Differences in NNMT mRNA expression were seen in the various regions. The highest levels of NNMT expression were in the spinal cord, medulla, and temporal lobe. The lowest levels of expression

TABLE 4
Relative Expression of NNMT mRNA in 16 Regions of the Human Brain

Brain region[a]	Ratio[b]
Subthalamic nucleus	1.00
Caudate nucleus	1.29
Cerebellum	1.55
Thalamus	1.79
Corpus callosum	2.26
Substantia nigra	2.53
Whole brain	2.85
Hippocampus	3.76
Cerebral cortex	4.26
Amygdala	4.59
Occipital lobe	6.20
Putamen	9.30
Frontal lobe	9.69
Temporal lobe	17.83
Medulla	26.75
Spinal cord	63.20

[a] Data are presented in ascending order of relative expression with the region with lowest expression being assigned a value of 1. [b] Ratios are expressed as ratio of NNMT:ß actin mRNA expression divided by subthalamic nucleus NNMT:ß actin ratio.

were in the cerebellum, caudate nucleus, and subthalamic nucleus. Detectable levels of the NNMT mRNA transcript were found in the substantia nigra.

SDS-PAGE/Western Analysis of Tissue Homogenates: NNMT was expressed in the spinal cord, temporal lobe, and liver. Highest expression was in the liver, followed by the spinal cord and then temporal lobe. NNMT was not expressed in human kidney cortex (data not shown). Caudate nucleus and cerebellum also expressed NNMT; the results are shown in Table 5. Both antibodies gave identical results.

NNMT Activity of Tissue Homogenates: NNMT activity was in accord with the expression of protein, with specific activities of 15.42 ± 0.98 in the liver, 0.25 ± 0.09 in the spinal cord, and 0.11 ± 0.12 in the temporal lobe, respectively. No activity was detected in the kidney homogenate. Results for caudate nucleus and cerebellum are shown in Table 5.

Cellular Localization of NNMT Protein: Both anti-NNMT antibodies gave identical results in the studies present below.

Temporal Lobe

Figure 1 shows the localization of NNMT in the temporal lobe (Fig. 1A) compared with no primary antibody control (Fig. 1B) and non-immune sheep serum (Fig. 1C). Staining was localized to the cell bodies of multipolar neurons and to the immediate area of the axonal and dendritic processes originating from the cell body. Preabsorption of the antibodies with antigenic peptide removed all evidence of staining (Fig. 1D). Preabsorption of the antibodies with the non-complementary antigenic peptide had no effect upon staining (data not shown).

Spinal Cord

Figure 2 shows the localization of NNMT in the spinal cord (Fig. 2A) compared with no primary antibody control (Fig. 2B) and non-immune sheep serum (Fig. 2C). Staining was expressed in the multipolar neurons. Preabsorption of the antibodies with antigenic peptide removed all evidence of staining (Fig. 2D). Preabsorption of the antibodies with non-complementary antigenic peptides had no effect upon staining (data not shown).

Cerebellum

Figure 3A–F shows the immunohistochemical staining for NNMT in both non-PD (Fig. 3B, C) and PD (Fig. 3D, E) cerebellum. Localization was limited overwhelmingly to the neurons of the granular layer, with staining evident only to a low degree in the molecular layer. No staining was evident in the white matter under any of the conditions used. No staining was evident when primary antibody was replaced with antibody diluent only (Fig. 3A). Preabsorption of the antibody with antigenic peptide removed all staining (Fig. 3F). Cerebella from PD subjects showed a greater degree of staining than those cerebella from the non-PD subjects. Figure 3M shows the result of GFAP staining of cerebellum. Localization of glial cells (astrocytic in morphology) was limited solely to the white matter and the inner layer of the granular

TABLE 5
Expression of NNMT Protein and Activity in Cerebellum and Caudate Nucleus

		Activity (U)[a]			Western Analysis (A.U.)[b]		
		Median	Min.	Max.	Median	Min.	Max.
Cerebellum	C	0.15	0.00	0.34	0.014	0.009	0.029
	PD	0.48	0.13	1.16	0.027	0.015	0.038
Caudate nucleus	C	0.00	0.00	1.43	0.026	0.012	0.056
	PD	1.79	0.15	5.55	0.052	0.04	0.076

[a] Expressed as specific activity (nmols product/hour/mg protein). [b] Expressed as absorbance units. Abbreviations: min., minimum; max., maximum.

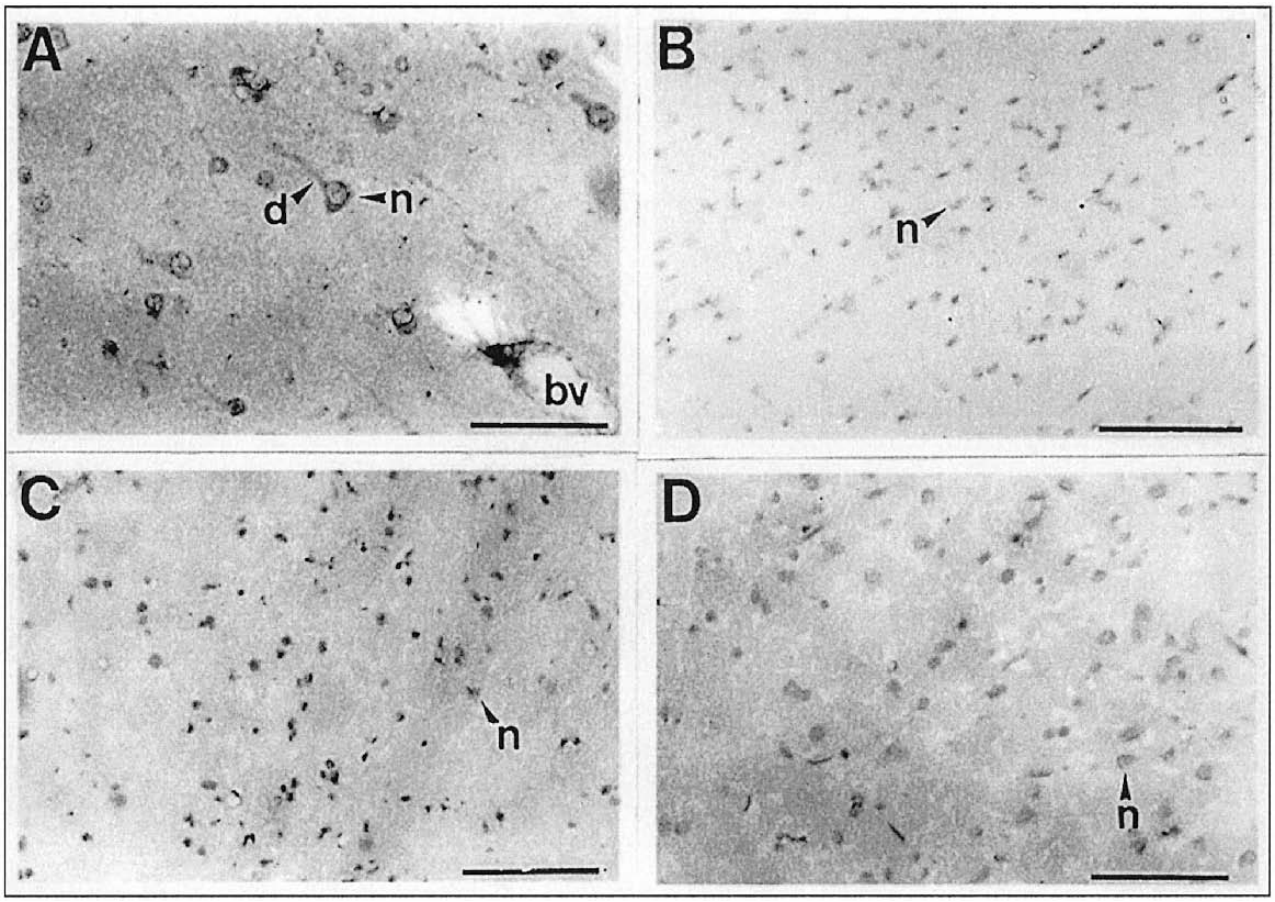

Fig. 1. Immunohistochemical localization of NNMT in temporal lobe. Sections (5 μm) were incubated with anti-NNMT (1:50, overnight, 4°C), followed by donkey-anti-sheep IgG (1:25, 1 h, room temperature) and goat PAP (1:50, 1 h, room temperature). Staining was developed using SigmaFAST DAB solution, counterstained in hematoxylin, and mounted in DPX mounting medium following dehydration in alcohol and xylene. A: Anti-NNMT a1. Abbreviations: n = neuron; d = dendritic process; bv = blood vessel; B: No primary antibody control. 1% (w/v) Bovine serum albumin-supplemented TBS was substituted for primary antibody. C: Non-immune sheep serum control. Non-immune sheep serum (1:50) was substituted for primary antibody. D: Preabsorbed anti-NNMT a1 antibody control. Anti-NNMT a1 antibody was incubated with an excess of antigenic peptide for 1 h at room temperature and substituted for primary antibody. Abbreviation: n = neuron. Magnification, ×250. Scale bar = 100 μm.

layer. No staining was observed in the majority of the granular layer, with limited expression in the molecular layer. GFAP staining was not associated with cell types that expressed NNMT. Expression of GFAP occurred in the same cell type in both PD and non-PD cerebellum. There was no staining associated with THO in the cerebellum (data not shown).

Midbrain Sections Containing Substantia Nigra and Third Nerve

Figure 3G–L shows the typical expression of NNMT in the substantia nigra/third nerve region of non-PD (Fig. 3G–I) and PD (Fig. 3J–L) subjects. In control subjects, NNMT was present in pigmented dopaminergic neurons of the substantia nigra and was not evident in glial cells (Fig. 3G, H). Expression was present in the cell body and the dendritic processes of the neurons. In the substantia nigra of subjects who had died from PD, expression was also neuronal but few pigmented neurons could be seen (Fig. 3J, L). Expression appeared limited to the cell bodies because axonal processes were absent or so small as to be difficult to detect. Preabsorption of the primary antibody removed all evidence of staining (data not shown). Staining was also observed in the third nerve-non-PD, with staining present in the cell body and along the length of the axonal process (Fig. 3I), however staining was barely detectable in this region in the PD material (Fig. 3K). Figure 3O shows the typical localization of dopaminergic neurons in the normal substantia nigra as shown using THO staining. Staining was present in the cell body, the axon, and the dendritic processes of the dopaminergic neuron. THO expression was associated with those cell-types that expressed NNMT. Figure 3N shows the typical localization of glial cells (astrocytic in morphology) in the substantia nigra as shown using GFAP staining. Expression of GFAP occurred in the same cell

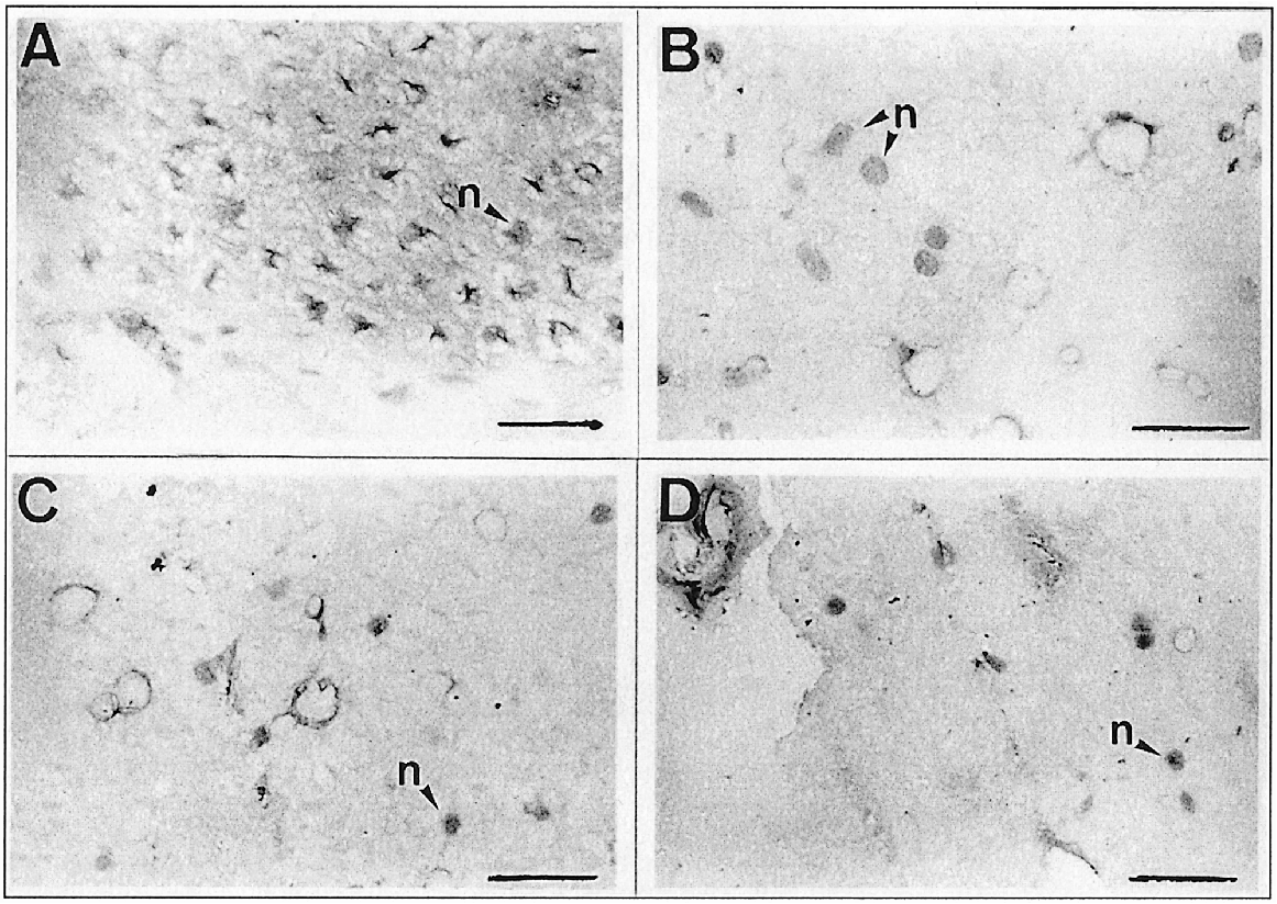

Fig. 2. Immunohistochemical localization of NNMT in spinal cord grey matter. NNMT was detected using anti-NNMT (1:100, 24 h, 4°C) followed by donkey-anti-sheep IgG (1:50, 1 h, room temperature) and goat PAP (1:100, 1 h, room temperature) and stained using SigmaFAST DAB. A: Anti-NNMT a2. B: No primary antibody control. C: Non-immune sheep serum control. D: Preabsorbed anti-NNMT a2 antibody control. Labeling is as described in (C). Magnification, ×400. Scale bar = 100 μm. Abbreviations: n = neuron.

type in both the PD and non-PD substantia nigra. GFAP staining was not associated with the cell types that expressed NNMT.

Caudate Nucleus

There was limited expression of NNMT in the cell bodies of neurons and faint expression could be seen in projections from these (data not shown). There was no obvious qualitative difference in the expression of NNMT between the PD and non-PD samples.

Liver

Figure 4 shows the localization of NNMT in human liver (Fig. 4A) compared with no primary antibody control (Fig. 4B) and non-immune sheep serum (Fig. 4C). NNMT was expressed in the periportal region only. In the portal triad, staining was present in the epithelia of the bile duct and the hepatic artery. Preabsorption of the antibodies with antigenic peptide removed all evidence of staining (Fig. 4D). Preabsorption of the antibodies with non-complementary antigenic peptide had no effect upon staining (data not shown).

Kidney

No NNMT expression was detectable in the kidney cortex (data not shown).

Stability of NNMT Protein upon Storage: There was no significant effect of storage upon the activity and protein stability of NNMT ($p > 0.05$ for each storage condition using a combination of Kruskal-Wallis non-parametric ANOVA and Dunn's Multiple Comparisons tests).

Quantitation of Expression of NNMT in the Brains of PD and Non-PD Subjects

Comparison of Protein Expression (Western Blotting) and Enzyme Activity in Caudate Nucleus and Cerebellum of Patients with PD and Non-PD Subjects: Table 5 summarizes the expression of NNMT in the caudate nucleus and cerebellum using an activity assay and densitometric analysis of the Western analysis. The levels of enzyme

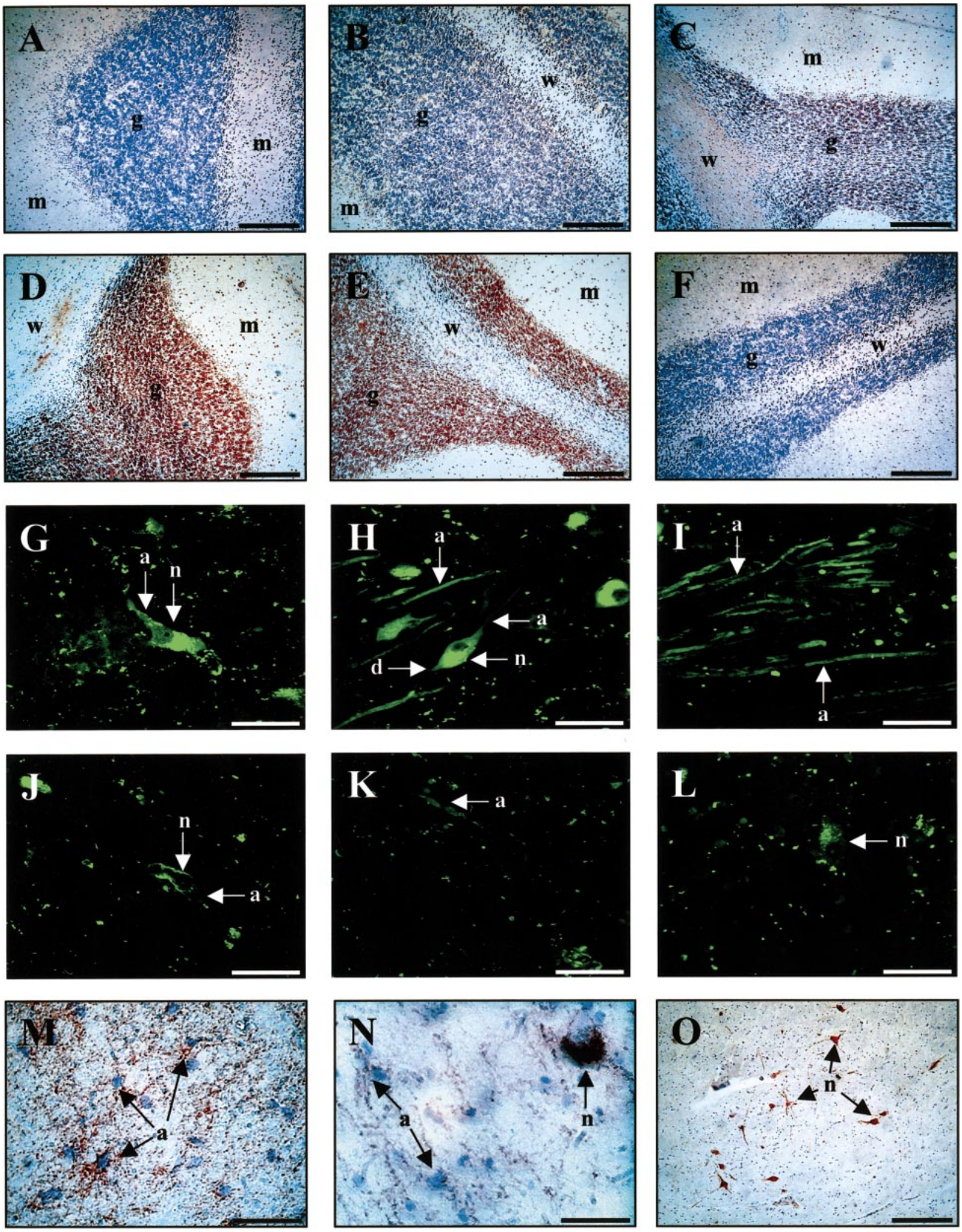

Fig. 3. Immunohistochemical staining of cerebella and substantia nigra. **A–F**: Immunohistochemical localization of NNMT protein in the cerebella of 2 controls and 2 PD cases typical of the expression observed. Flash-frozen sections (12 μm) were probed for NNMT expression using anti-NNMT a1 as described in Figure 1. NNMT protein localization is shown by brown

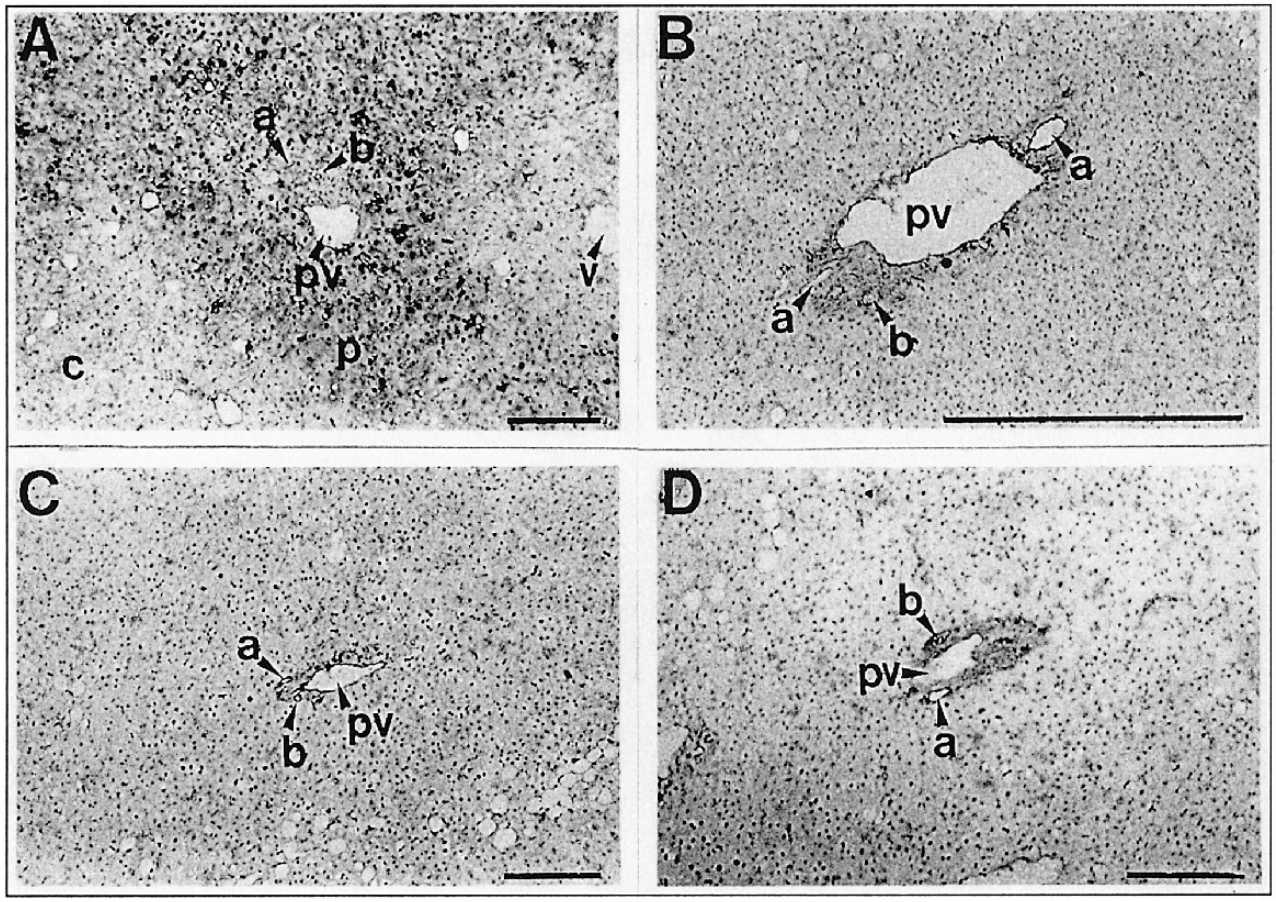

Fig. 4. Immunohistochemical localization of NNMT in liver. NNMT was detected using anti-NNMT (1:100, overnight, 4°C) followed by donkey-anti-sheep IgG (1:50, 1 h, room temperature) and goat PAP (1:100, 1 h, room temperature) and stained using SigmaFAST DAB. A: Anti-NNMT a1. Abbreviations: a = hepatic artery; b = bile duct; pv = hepatic portal vein; cv = central vein; p = periportal hepatocytes; c = centrilobular hepatocytes. Magnification, ×78.75. Scale bar = 100 μm. B: No primary antibody control. Labeling is as described in (A). Magnification, ×250. Scale bar = 100 μm. C: Non-immune sheep serum control. Labeling is as described in (A). Magnification, ×78.75. Scale bar = 100 μm. D: Preabsorbed anti-NNMT a1 antibody control. Labeling is as described in (A). Magnification, ×100. Scale bar = 100 μm.

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DAB staining; blue staining is hematoxylin counterstain. A: No primary control. B: Control 97.0700. C: Control 94.0485. D: PD 96.0597. E: PD 97.0681. F: Preabsorbed anti-NNMT a1 antibody control. Abbreviations: g = granular layer; m = molecular layer; w = white matter. Magnification, ×100. Scale bar = 100 μm. **G–L**: Immunohistochemical localization of NNMT protein in the substantia nigra of 2 controls and 2 PD subjects typical of the expression observed. Flash-frozen sections (12 μm) were probed for NNMT expression using anti-NNMT a2 (1:50) for 1 h at room temperature followed by donkey-anti-sheep IgG fluorescein conjugate (1:50) for 1 h at room temperature. G: Control 97.0700 substantia nigra. H: Control 97.0701 substantia nigra. I: Control 97.0701 third nerve. J: PD 97.0681 substantia nigra. K: PD 07.0681 third nerve. L: PD 96.0597 substantia nigra. Abbreviations: n = neuron cell body; d = dendritic processes; a = axonal process. Magnification, ×400. Scale bar = 25 μm. **M, N**: Typical expression of glial cells in the cerebellum and substantia nigra respectively. Samples were probed for GFAP using anti-GFAP (1:100) for 1 h at room temperature followed by donkey-anti-sheep IgG biotin conjugate (1:50) followed by streptavidin-conjugated horseradish peroxidase (1:50) for 1 h each. Abbreviations: a = astrocytes; n = pigmented dopaminergic neuron showing neuromelanin pigmentation. Magnification, ×400. Scale bar = 25 μm. O: Immunohistochemical localization of THO in control substantia nigra. Samples were probed for THO using anti-THO (1:20) for 1 h at room temperature followed by secondary and tertiary antibodies as described in (M and N). Abbreviations: n = pigmented dopaminergic neurons. Magnification, ×100. Scale bar = 100 μm.

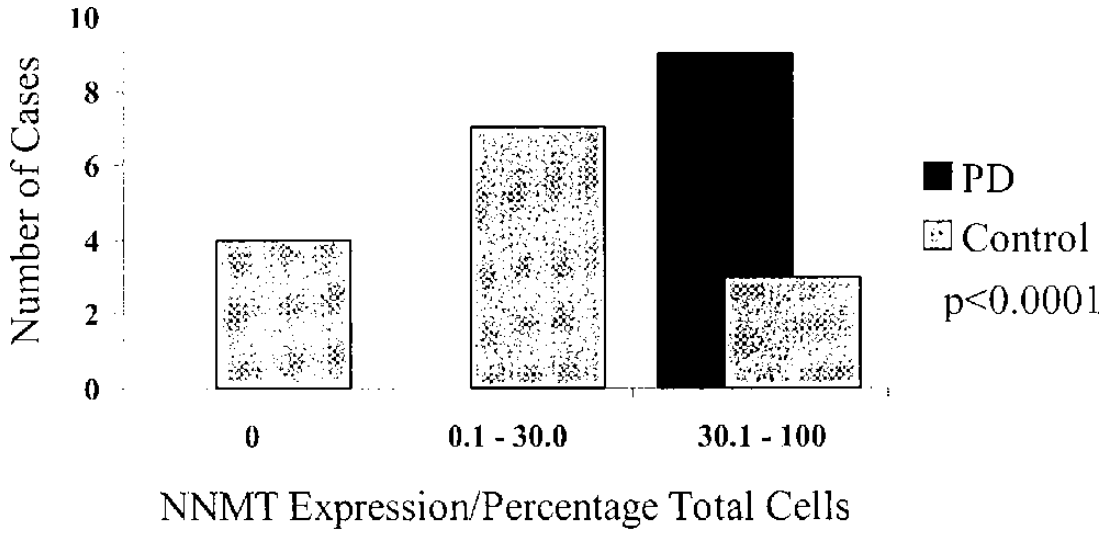

Fig. 5. Frequency distribution of the expression of NNMT in the granular neurons of both non-PD and PD cerebella. Non-PD cerebella followed a 1:2:1 distribution of activity, with a median of 5% and a range of zero to 63%. PD cerebella were all in the high expressor (>30.1% expression) group, with a median of 95.5% and a range of 88.0–97.9%. The PD group showed significantly higher expressors than the non-PD group ($p < 0.0001$ using a non-parametric Mann-Whitney *U*-test). Grey bars = non-PD; black bars = PD.

activity varied greatly between subjects, with those who had died from PD having significantly higher enzyme activity than non-PD subjects ($p = 0.03$ for caudate nucleus, $p = 0.029$ for cerebellum using the Mann-Whitney *U* non-parametric test). The levels of NNMT protein expression in subjects who had died from PD was significantly higher than non-PD subjects ($p = 0.0326$ for caudate nucleus, $p = 0.0415$ for cerebellum using the Mann-Whitney *U* non-parametric test).

Comparison of Protein Expression (Immunohistochemistry) in Cerebellum of Subjects with PD and Non-PD Subjects: The percentage of granular cells expressing NNMT in control brains ranged from zero to 63%, with a median of 5%. Assignment of results into 3 arbitrary groups: zero; >0–30%; and >30% gave the distribution shown in Figure 5. This distribution in the control group is similar to the 1:2:1 distribution of the level of NNMT protein expression seen in normal human liver samples (11). The PD group was comprised entirely of high expressors (>30.1% of granular cells expresssing the protein), with a median of 95.5% and range of 88%–97.9%. Statistical analysis of the PD and non-PD groups using a two-tailed non-parametric Mann-Whitney *U*-test showed that the 2 groups were highly significantly different ($p < 0.0001$). There was no significant difference between the counting of the 2 investigators, the average difference of the means being 2% with SD of 2.3%.

There were no significant differences between PD and non-PD subjects for postmortem interval, pH of cerebellum, and age at death. No significant trend was observed between the level of NNMT expression in the cerebellum and any of the above parameters in PD subjects (data not shown). One significant trend was found in that the percentage of granular cells expressing NNMT was inversely correlated with disease duration ($p = 0.03$, $r = -0.73$ using Spearman rank correlation analysis).

Comparison of NNMT Expression in the Substantia Nigra of PD and Non-PD Subjects: As seen in this study, expression of NNMT was limited to dopaminergic neurons, identified by morphology and the presence of neuromelanin. Significantly fewer dopaminergic neurons per unit area were seen in the PD tissue samples than in non-PD cases (7.79 control, 3.42 PD; $p = 0.004$ using the Student *t*-test), although the percentage of neurons expressing NNMT was not significantly different in the 2 groups. In sections counterstained with propidium iodide many more glial cell nuclei were evident in PD samples compared with the numbers in non-PD tissue.

DISCUSSION

Characterization of NNMT Expression and Antibody Specificity

In order to determine whether NNMT may be linked with the occurrence of PD, we began by determining the expression of NNMT in normal brain tissue using a combination of Northern blotting, Western blotting, immunohistochemistry, and activity assay. The specificity of the antibody used in 2 of these techniques, which previously had been shown to precipitate human liver NNMT activity specifically, was further confirmed by its failure to detect NNMT activity in the renal cortex, a tissue region known not to exhibit enzymic activity (18), and its specific localization of NNMT immunoreactivity

in the periportal region of liver. Using this approach, we have demonstrated that NNMT is differentially expressed regionally in the brain and expression occurs only in neurons. This is not unique, as many enzymes have been shown to have distinct regional distributions (20–22). Non-uniform distribution may have functional significance and could represent regional requirements for either 1-methylnicotinamide or clearance of the parent compound.

Use of Extra-Substantia Nigra Tissue for Assessing NNMT Expression in PD

The use of substantia nigra tissue in studies of PD such as this is complicated by the reduction in the number of pigmented dopaminergic neurons present due to the degeneration process of the disease. Thus, in the case of an enzyme chiefly expressed in neurons, there could be a reduction in its expression simply because of the death of neurons rather than any specific effect on gene regulation. We clearly show in the substantia nigra of subjects who did not have PD that NNMT is expressed in the dopaminergic neurons and neurons of the third nerve. The enzyme is not limited to the cell body, but axonal transport of NNMT occurs. These are neurons that die in PD, and our results show the near absence of these cells in PD brain sections. Thus, the substantia nigra was considered not to be a useful tissue to study because the brain samples could only be obtained from patients at the end-stage of the disease when neuronal destruction was at a maximum. Therefore, the use of extra-substantia nigra tissues, namely the cerebellum and caudate nucleus, a component of the basal ganglia, which are not strongly associated with the neurodegenerative processes in PD, would seem more appropriate to investigate levels of specific protein expression. Furthermore, in this study these regions were shown to have low levels of NNMT mRNA. In view of our starting hypothesis that PD was associated with increased levels, these regions were deemed useful because it would be relatively easy to detect an increase starting from a low normal baseline. Our results clearly demonstrated that there were significantly higher levels of NNMT in these brain regions in PD patients compared with the same regions in control brains. This was confirmed using 3 separate techniques, a finding that was not apparent from using substantia nigra tissue. It might be argued that, because caudate nucleus contains projections from dopaminergic neurons of the substantia nigra, and we demonstrated NNMT in these projections, there should be lower levels of NNMT in this region of the brain in PD subjects. However, the contribution to total caudate nucleus NNMT of the protein in these projections is minimal. Therefore, this did not influence the findings of the Western blotting and activity assays.

Comparison of NNMT Expression in Non-Parkinsonian and Parkinsonian Cerebellum

The findings of elevated levels of NNMT in specific, unaffected brain regions of PD sufferers is in accord with the findings of Matsubara et al (23), who found increased levels of NNMT in the cerebrospinal fluid of parkinsonian patients. The higher levels of NNMT expression in PD cerebella may be due to either (a) differences in the genetically determined levels of constitutive expression, (b) linked to the onset of disease, or (c) secondary factors such as the effects of drug therapy. Furthermore, it should be borne in mind that the numbers of subjects studied are quite small, although these represent a major proportion of the available tissue from 1 major brain bank in which 3 types of investigation (SDS-PAGE/Western blotting, immunohistochemistry, and enzymic activity determination) could be carried out. This therefore suggests that a larger scale investigation is required. Notwithstanding these caveats, the significant inverse correlation between NNMT expression in the cerebellum and disease duration is suggestive of a causal link between enzyme and disease.

The assertion that levels of NNMT expression are higher in PD compared with levels in tissue from control subjects raises the questions of what is meant by a control subject and how closely do these levels represent the vivo situation in health, given that all the subjects were, of course, dead. None of the control subjects died of cancer and, therefore, did not receive cytotoxic chemotherapy that might have grossly disrupted cellular protein synthesis. The nearest answer to the second question was sought in the comparison of NNMT expression in the cerebellum of these control subjects with that seen in the livers of young healthy adults dying violent deaths in road traffic accidents (RTAs) and whose livers were rapidly removed for transplantation (11). This latter was considered to represent the nearest to the normal in vivo situation it is possible to approach with human subjects. Both groups have levels of expression that could be represented as having a 1:2:1 distribution. Furthermore, the low and intermediate expression group had levels that were not greatly dissimilar, whereas the high expressor groups had well separated from the other two. Thus both the levels in the RTA liver group and the control cerebella reported here had similar distributions to the large population study reported by Rini et al (10). Therefore, our claim that our control data represent the situation in the normal human brain is not without foundation and the same genetic factor responsible for determining liver activity appears to operate in the brain also.

Despite the above reservations on the effects of treatment, particularly bearing in mind that NNMT is an inducible enzyme (11), it is worthwhile considering how genetically determined high levels of NNMT expression

might be related to the onset of disease. Two main reasons can be postulated: (i) the production of toxic methylpyridinium ions and (ii) reduced substrate availability for Complex I. In addition, sub-optimal levels of vitamin B3 may promote apoptosis because nicotinamide was been shown to inhibit apoptosis induced by tertiary butylhydroperoxide (24) and methyl-phenyl-tetrahydropyridine (MPTP) (25).

Increased Methylpyridinium Ion Analogues

As previously described, NNMT, in addition to metabolizing nicotinamide, metabolizes structurally related compounds. For example, 4-phenylpyridine, found in low amounts in tea and green leafy vegetables, can be converted to MPP^+ by NNMT (12). Once formed within the brain, methylpyridinium compounds can migrate to any region where they may be taken up by the dopaminergic reuptake system of dopaminergic neurons. The pigmented neurons of the substantia nigra are particularly susceptible to this due of the high levels of neuromelanin that they contain because of adsorption to neuromelanin. Thus, over a long period of time a toxic dose could build up, leading to neurodegeneration and the onset of PD. In addition, NNMT can convert distant structural analogues of endogenous origin, e.g. ß-carbolines and tetrahydroisoquinolines, to pyridinium derivatives. Increased enzymic N-methylation of such compounds in PD has been reported (26, 27), although what the relationship between NNMT and the enzyme(s) that methylates ß-carbolines and tetrahydroisoquinolines remains to be elucidated, although Gearhart et al postulated that the methylation of all 3 classes of compounds could be brought about by the same entity (28).

Complex I Activity

NADH is the electron donor for Complex I oxidation of glutamate and pyruvate that forms 1 of the 2 starting points of the electron transport chain of mitochondrial oxidative phosphorylation. Reduced Complex I activity has been shown to be linked with several neurological diseases such as tardive dyskinesia (29), Leigh syndrome (30), and PD (31, 32), and overt vitamin B3 deficiency causes neurological pathology (33, 34). The recent elucidation of the role of parkin in the predominantly Japanese form of familial PD also re-emphasizes the potential role of energy deficiency in the etiology of sporadic PD (35, 36). The absence of Lewy bodies in this form of familial PD is attributed to the inability of the mutant parkin to bring about α-synuclein ubiquitinylation. Clearly in sporadic PD α-synuclein is ubiquitinylated. Because it forms a major component of Lewy bodies (37, 38), α-synuclein in sporadic PD is not undergoing proteasomal catabolism efficiently. Numerous studies have shown that α-synuclein, parkin, and ubiquitin terminal hydroxylase mutations are rare, if ever, causes of sporadic PD (39–41); hence mutation of these genes would appear an unlikely cause of inefficient α-synuclein catabolism. Mutations of genes whose products go to form the proteasome are a possible but somewhat unexplored cause, as is relative deficiency of the Complex 1 substrate-NADH. Although tryptophan metabolism can yield vitamin B3, this is a very inefficient source and Western diets tend to have barely adequate levels of intake. It would seem logical, therefore, that an elevated level of NNMT would place higher dietary requirements for both vitamin B3 and tryptophan, which, if not fulfilled, may lead to a reduction in available NADH and thence to decreased Complex I activity. Thus, a subsequent insult that further reduced Complex I activity could then lead to oxidative stress and its further consequences, particularly in cells such as the dopaminergic neurons. Because of such considerations NADH therapy has been proposed as a treatment for PD. Pilot studies gave mixed results but no large-scale multicenter trial has been carried out (42).

Some of the effects of the genetically high level of NNMT could be offset by high dietary intake of vitamin B3, although nicotinamide induces NNMT expression in the liver and possibly other tissues (43). High nicotinamide levels give rise to increased choline synthesis (44–47), although the effect on choline levels is marginal. Nevertheless, this may not be beneficial in brains that are developing PD.

In conclusion, NNMT is a neuronally expressed enzyme with a wide substrate profile. In the liver, levels of activity are strongly genetically determined. Depending on substrate exposure, the products of its activity may be either directly toxic to Complex I or cause a diminution of available levels of the essential electron donor for this complex's action. In view of the fact that reduced Complex I activity is one of the cardinal biochemical features of PD, the findings of high levels of this enzyme in the cerebella and caudate nucleus of PD patients, and the inverse correlation of enzyme expression in the cerebellum with disease duration suggest that this enzyme may be one link between the genetic and environmental factors in the etiology of PD.

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REFERENCES

1. Hoshino J, Kuhne U, Kroger H. Methylation of nicotinamide in rat liver cytosol and its correlation with hepatocellular proliferation. Biochim Biophys Acta 1982;719:518–26
2. Kang-Lee Y, McKee RW, Wright SM, Swendseid ME, Jenden DJ, Jope RS. Metabolic effects of nicotinamide administration in rats. J Nutr 1983;113:215–21

3. Nakagawa K, Miyazaki M, Okui K, Kato N, Moriyama Y, Fujimura S. N1-methylnicotinamide level in the blood after nicotinamide loading as further evidence for malignant tumour burden. Jap J Cancer Res 1991;82:1277–83
4. Naoi M, Dostert P, Yoshida M, Nagatsu T. N-methylated tetrahydroisoquinolines as dopaminergic neurotoxins. Adv Neurol 1993; 60:212–17
5. Hanazawa Y, Sato K, Kuroiwa N, Ogawa M, Kuriyama A, Asanagi M, et al. Characterisation of nicotinamide methyltransferase in livers of mice bearing Ehrlich ascites tumours: Preferential increase of activity. Tumour Biol 1994;15:7–16
6. Naoi M, Matsura S, Takahashi T, Nagatsu T. A N-methyltransferase in human brain catalyses N-methylation of 1,2,3,4-tetrahydroisoquinoline into the N-methyl-1,2,3,4-tetrahydroisoquinoline, a precursor of a dopaminergic neurotoxin, N-methylisoquinolinium ion. Biochem Biophys Res Commun 1989;161:1231
7. Naoi M, Maruyama W, Niwa T, Nagatsu T. Novel toxins and Parkinson's disease: N-methylation and oxidation as metabolic bioactivation of neurotoxins. J Neur Transm-Suppl 1994;41:197–205
8. Aksoy S, Szumlanski CL, Weinshilboum RM. Human liver nicotinamide N-methyltransferase: cDNA cloning, expression and biochemical characterisation. J Biol Chem 1994;269:14835–40
9. Aksoy S, Brandriff BF, Ward A, Little PF, Weinshilboum RM. Human nicotinamide N-methyltransferase gene: Molecular cloning, structural characterisation and chromosomal localisation. Genomics 1995;29:555–61
10. Rini JN, Szumlanski CL, Guerciolini R, Weinshilboum RM. Human liver nicotinamide N-methyltransferase: Ion-pairing radiochemical assay, biochemical properties and individual variation. Clin Chim Acta 1989;186:359–74
11. Smith ML, Burnett D, Benett P, et al. A direct correlation between nicotinamide N-methyltransferase activity and protein levels in human liver cytosol. Biochim Biophys Acta 1998;1422:238–44
12. Ansher SS, Cadet JL, Jakoby WB, Baker JK. Role of N-methyltransferases in the neurotoxicity associated with the metabolites of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and other 4-substituted pyridines present in the environment. Biochem Pharmacol 1986;35:3359–63
13. Ziegler M. New functions of a long-known molecule. Emerging roles of NAD in cellular signalling. Eur J Biochem 2000;267:1550–64
14. Parsons RB, Ramsden DB, Waring RH, Barber PC, Williams AC. Hepatic localisation of rat Cysteine Dioxygenase. J Hepatol 1998; 29:595–602
15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Analyt Biochem 1976;72:248–54
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–85
17. Sternberger LA, Hardy PH Jr, Cuculis JJ. The unlabelled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-anti-horseradish peroxidase) and its use in the identification of spirochetes. J Histochem Cytochem 1970;18:315
18. Sano A, Endo N, Takitani S. Fluourometric assay of rat tissue N-methyltransferases with nicotinamide and 4 isomeric methylnicotinamides. Chem Pharmaceut Bull 1992;40:153–56
19. Kingsbury AE, Foster OJF, Nisbet AP, et al. Tissue pH as an indicator of messenger RNA preservation in human post-mortem brain. Mol Brain Res 1995;28:311–18
20. Jiang H, Ho S-L, Xie T, Young LP. Distribution of catechol O-methyltransferase (COMT) expression in human central nervous system. Mov Dis 1998;13Suppl. 2:89
21. Parsons RB, Barber PC, Waring RH, Williams AC, Ramsden DB. Cysteine dioxygenase: Regional localisation of activity in rat brain. Neurosci Lett 1998;248:101–4
22. Parsons RB, Waring RH, Williams AC, Ramsden DB. Cysteine dioxygenase: Regional localisation of protein and mRNA in rat brain. J Neurosci Res 2001;65:78–84
23. Akoyama K, Matsubara K, Kondo K, et al. Nicotinamide-N-methyltransferase is higher in the lumbar cerebrospinal fluid of patients with Parkinson's disease. Neurosci Lett 2001;298:78–80
24. Klaidman LK, Mukherjee SK, Hutchin TP, Adams JD. Nicotinamide as a precursor for NAD^+ prevents apoptosis in the mouse brain induced by tertiary-butylhydroperoxide. Neurosci Lett 1996; 206:5–8
25. Mukherjee SK, Klaidman LK, Yasharel R, Adams JD. Increased brain NAD prevents neuronal apoptosis in-vivo. Eur J Pharmacol 1997;330:27–34
26. Muller T, Sallstrom Baum S, Haussermann P, Woitalla D, Rommelspacher H, Przuntek H, et al. Plasma levels of R- and S-salsolinol are not increased in ''de novo'' Parkinsonian patients. J Neur Transm 1998;105:239–46
27. Nagatsu T. Isoquinoline neurotoxins in the brain and Parkinson's disease. Neurosci Res 1997;29:99–111
28. Gearhart DA, Collins MA, Lee JM, Neafsey EJ. Increased ß-carboline N-methyltransferase activity in the frontal cortex in Parkinson's disease. Neurobiol Dis 2000;7:201–11
29. Goff DC, Tsai G, Beal MF, Coyle JT. Tardive dyskinesia and substrates of energy metabolism in CSF. Amer J Psychiatr 1995;152: 1730–36
30. Taylor MJ, Robinson BH. Evoked potentials in children with oxidative metabolic defects leading to Leigh syndrome. Pediat Neurol 1992;8:25–29
31. Mann VM, Cooper JM, Daniel SE, et al. Complex I, iron and ferritin in Parkinson's disease substantia nigra. Ann Neurol 1994;36: 786–881
32. Schapira AH. Mitochondrial complex I deficiency in Parkinson's disease. Adv Neurol 1993;60:288–91
33. Gibson GE, Blass JP. Oxidative metabolism and acetylcholine synthesis during acetylpyridine treatment. Neurochem Res 1985;10: 453–67
34. Sanada H, Nakashima Y, Utsuki Y, Kawada S. Effect of niacin deficiency on the metabolism of brain amines in rats. J Nutr Sci Vitaminol 1978;24:159–66
35. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392: 605–8
36. Schlossmacher MG, Hattori N, Frosch MP, et al. Ubiquitination of a new form of α-synuclein by parkin from human brain: Implications for Parkinson's disease. Science 2001;13:263–69
37. Irizarry MC, Growdon W, Gomez-Isla T, et al. Nigral and cortical Lewy bodies and dystrophic nigral neurites in Parkinson's disease and cortical Lewy body disease contain α-synuclein immunoreactivity. J Neuropathol Exp Neurol 1998;57:334–37
38. Li K, Ito H, Tanaka H, Hirano A. Immunocytochemical co-localisation of the proteosome in ubiquinated structured in neurodegenerative disease and the elderly. J Neuropathol Exp Neurol 1997;56: 125–31
39. Vaughan J, Durr A, Tassin J, et al. The α-synuclein Ala53Thr mutation is not a common cause of familial Parkinson's disease: A study of 230 European cases. European Consortium on Genetic Susceptibility in Parkinson's Disease. Ann Neurol 1998;44:270–73
40. Wang M, Hattori N, Matsumine H, et al. Polymorphism in the parkin gene in sporadic Parkinson's disease. Ann Neurol 1999;45:655–58
41. Lincoln S, Vaughan J, Wood N, et al. Low frequency of pathogenic mutations in the ubiquitin carboxy-terminal hydrolase gene in familial Parkinson's disease. Neuroreport 1999;10:427–29
42. Birkmayer JG, Vrecko C, Vole D, Birkmayer W. Nicotinamide adenine dinucleotide (NADH)—a new therapeutic approach to

Parkinson's disease. Comparison of oral and parenteral application. Acta Neurol Scand Suppl 1993;146:32–35
43. Smith ML. Neurotoxic pyridine derivatives and the role of methylation. PhD Thesis, The University of Birmingham, UK.
44. Erb C, Klein J. Enhancement of brain choline levels by nicotinamide: Mechanism of action. Neurosci Lett 1998;249:111–14
45. Erb C, Seidel A, Frank H, Platt KL, Oesch F, Klein J. Formation of N-methylnicotinamide in the brain from a dihydropyridine-type drug: Effect on brain choline. Biochem Pharmacol 1999;57:681–84
46. Koppen A, Klein J, Holler T, Loffelholz K. Synergistic effect of nicotinamide and choline administration on extracellular choline levels in the brain. J Pharmacol Exper Therap 1993;266:720–25
47. Vargas HM, Jenden DJ. Elevation of cerebrospinal fluid choline levels by nicotinamide involves the enzymatic formation of N1-methylnicotinamide in brain tissue. Life Sci 1996;58:1995–2002

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