Parkinson’s disease-associated parkin colocalizes with Alzheimer’s disease and multiple sclerosis brain lesions

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Abstract

Parkin is implicated in the pathogenesis of Parkinson’s disease. Furthermore, parkin targets misfolded proteins for degradation and protects cells against various forms of cellular stress, including unfolded-protein and oxidative stress. This points towards a protective role of parkin in neurological disorders in which these stressors are implicated, including Alzheimer’s disease (AD) and multiple sclerosis (MS). Here, we assessed parkin distribution in AD and MS brain tissue using immunohistochemistry. In AD brains, parkin colocalized with classic senile plaques and amyloid-laden vessels as well as astrocytes associated with both lesions. Similarly, we observed enhanced astrocytic parkin immunoreactivity in MS lesions, particularly in inflammatory lesions. Furthermore, parkin mRNA expression was increased in an astrocytoma cell line after free radical exposure. Our data indicate that parkin is upregulated in AD and MS brain tissue and might represent a defense mechanism to counteract stress-induced damage in AD and MS pathogenesis.

Keywords
Alzheimer’s disease; astrocytes; multiple sclerosis; oxidative stress
Introduction

The parkin gene is linked to the development of familial Parkinson’s disease (PD) and maps to the long arm of chromosome 6 (6q25.2-q27).\(^1\)\(^,\)\(^2\) It encodes a 52-kDa protein that functions as an E3 ubiquitin ligase, targeting unfolded and damaged proteins for proteasomal degradation.\(^3\)\(^,\)\(^4\) In addition, parkin is directly involved in neuroprotection by regulating stress-related pathways.\(^5\)\(^,\)\(^6\) Recently, parkin has also been implicated in protecting cells from mitochondrial-associated cell death.\(^7\) Taken together, there is ample evidence that parkin exerts neuroprotective effects, particularly in PD pathology. Remarkably, little is known about the distribution of parkin in other neurodegenerative and neuroinflammatory disorders, and its putative protective role in these neurological conditions.

Alzheimer’s disease (AD) is the most common form of dementia, and is characterized pathologically by senile plaques (SPs), neurofibrillary tangles (NFTs) and cerebral amyloid angiopathy (CAA).\(^8\) Both SPs and CAA consist primarily of aggregated protein deposits of the amyloid-beta protein (A\(\beta\)), whereas NFTs mainly consist of intracellular accumulated hyperphosphorylated tau protein.\(^9\)\(^,\)\(^10\) The accumulation of A\(\beta\) and tau characterizes AD as a protein misfolding disease, suggesting a pathogenic role for defective protein clearance by the ubiquitin-proteasome system. Indeed, ubiquitin is abundantly present in both SPs and NFTs\(^11\) and overall proteasome function is impaired in AD brains.\(^12\) Furthermore, misfolded A\(\beta\) is considered to be the key mediator of cellular oxidative stress in AD,\(^13\) and ample evidence exists that oxidative stress is central to neurodegeneration in AD.\(^14\)\(^,\)\(^15\)

Multiple sclerosis (MS) is the most common nontraumatic neurological disease in young adults, and is characterized by inflammation and demyelination in the central nervous system (CNS). Traditionally, MS has been regarded as a neuroinflammatory disorder, however evidence is accumulating that neuroaxonal pathology can be extensive in the chronic phase of the disease.\(^16\) The pathological pathways leading to axonal degeneration in MS are still not fully understood, but evidence points to two tightly interrelated mechanisms: Ca\(^{2+}\) toxicity and energy dysbalance, which both lead to mitochondrial dysfunction and oxidative stress (for review, see\(^16\)).

Although the pathogenetic mechanisms upstream of cellular stress processes might be different in AD and MS, cellular oxidative stress leading to neurodegeneration is a common denominator in both diseases.\(^17\)\(^,\)\(^18\) As such, parkin might be involved in the pathogenesis of AD and MS. In fact, it has been demonstrated that parkin significantly protects cells from mitochondrial toxins and intracellular A\(\beta\) accumulation\(^7\) and clearance.\(^19\) Although these findings suggest that parkin is involved in pathogenic mechanisms underlying AD and MS, little is known about the expression of parkin in AD and MS brain tissue. Therefore, using immunohistochemistry, we analyzed the distribution of parkin in MS and AD brains, and investigated its possible association with the characteristic lesions of both neurological diseases. Moreover, we assessed parkin mRNA expression in human astrocytoma cells after tert-butyl hydroperoxide (tbH\(_2\)O\(_2\)) and A\(\beta\)\(_{1-42}\) treatment.

Materials and Methods

Brain tissue

Temporal neocortex tissue samples from 10 AD patients (age 71.3 ± 9.2 years; post mortem delay 6.1 ± 1.8 hours), 5 AD patients with CAA, and 5 control subjects without neurological disease (age 71 ± 21 years; post mortem delay 6.6 ± 0.5 hours) were obtained.
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after rapid autopsy and immediately frozen in liquid nitrogen (The Netherlands Brain Bank, Amsterdam, The Netherlands). White matter samples from 14 MS patients (age 56.9 ± 10.6; post mortem delay 8.2 ± 2.1 hours) and 4 control patients (age 83.7 ± 6.7; post mortem delay 6.3 ± 1.9 hours) were obtained after rapid autopsy and immediately frozen in liquid nitrogen (The Netherlands Brain Bank, Amsterdam, The Netherlands). White matter MS tissue samples were selected using post-mortem magnetic resonance imaging as published previously.20

The diagnosis of AD was based on a combination of neuropathological and clinical criteria.21 Table 1 provides an overview of the diagnosis, Braak & Braak score, CAA grading, age, post-mortem interval and gender of the AD patients used in this study. CAA grading was established by quantification of the number of Aβ-positive vessels in one microscopic field (magnification 2.5x), as described in a previous report.22 At least 4 microscopic fields of temporal neocortex were analyzed and categorized as follows: 0 (-, no CAA), 0-10 (+, sparse CAA), 10-20 (++, moderate CAA) and >20 (+++, severe CAA) vessels affected by Aβ deposition. Table 2 provides a summary of MS patient details. MS lesions were detected and classified by immunohistochemical analysis using antibodies directed against proteolipid protein (PLP), a myelin component, and anti-major histocompatibility complex class II, clone LN3, as described previously.23,24 In short, active lesions are characterized by abundant perivascular and parenchymal leukocyte infiltration, chronic active lesions are characterized by a rim of activated microglia and macrophages and a non-inflammatory center and chronic inactive lesions are hypocellular and contain few inflammatory cells.

Cell culture

The human astrocytoma cell line U373 MG (ATCC, Manassas, VA Rockville, MD), which is an established model for studies on astrocytes,25 was used. Cells were plated as a monolayer in poly-L-lysine (Sigma, Vienna, Austria)-coated culture flasks (Greiner Bio-One, Frickenhausen, Germany) and grown in DMEM/HAM-F10 (1:1, Life Technologies, Vienna, Austria) containing 10% foetal calf serum (FCS, Life Technologies), 2 mM l-glutamin (Life Technologies), and penicillin/streptomycin (50 mg/ml; Life Technologies).

Immunohistochemistry

Immunohistochemistry was used to detect parkin immunoreactivity in temporal neocortex and white matter in AD, MS and control subjects. Cryosections (5 µm) were air-dried and fixed in acetone for 10 minutes. Then, sections were incubated with a well-characterized goat-anti-parkin (1:2000; Millipore, Billerica, MA, USA26,27) for 60 minutes at room temperature. Subsequently, sections were incubated with biotin-labeled rabbit-anti-goat (DAKO, Glostrup, Denmark) for 60 minutes followed by s-ABC-HRP complex (1:200; DAKO, Glostrup, Denmark). Diaminobenzidine tetrachloride (DAB; Sigma, St. Louis, MO, USA) was used as chromogen. Finally, sections were counterstained with haematoxylin and mounted with DEPEX (Gurr, BDH Laboratories, Poole, UK). Between all incubation steps, sections were extensively washed in phosphate-buffered saline (PBS, pH 7.4). All antibodies were diluted in PBS containing 1% bovine serum albumin and 0.05% Tween-20, which also served as a negative control. Negative controls were essentially blank. Congo red staining of AD tissue was performed as described in a previous report.22
Double immunofluorescence

For colocalization studies, sections were incubated for 60 minutes at room temperature with anti-parkin antibody together with an antibody directed against glial fibrillary acidic protein (Z0334, GFAP; 1:500; DAKO, Glostrup, Denmark), major histocompatibility complex class II (MHC-II; 1:50; DAKO, Glostrup, Denmark), Aβ (ab 10148, 1:400; Abcam, Cambridge, UK) or hyperphosphorylated tau (AT8, 1:1000; Thermoscientific, Rockford, IL, USA). Sections were simultaneously incubated with the primary antibodies of interest. Biotin-labeled donkey anti-goat antibody coupled to Alexa594 (1:400, Molecular Probes, The Netherlands), biotin-labeled donkey anti-rabbit antibody coupled to Alexa488 (1:400, Molecular Probes, The Netherlands) and rabbit-anti-mouse F(ab')2 (1:500; DAKO, Glostrup, Denmark) followed by alexa 594-labeled streptavidin (1:400, Molecular probes, Leiden, The Netherlands) were used as secondary antibodies. Between all incubation steps, sections were extensively washed in PBS (pH 7.4). Sections were analyzed using Leica TCS SP2 AOPS confocal laser scanning microscope and Leica DM6000 B digital microscope (Leica Microsystems, Rijswijk, The Netherlands). All antibodies were diluted in PBS containing 1% bovine serum albumin and 0.05% Tween-20, which also served as a negative control. Negative controls were essentially blank.

Quantification of parkin staining in normal vessels and CAA.

Investigation of colocalization of parkin immunoreactivity with amyloid-laden vessels was performed as follows: of each patient, a Haematoxylin-Eosin (HE) staining was performed to determine the total number of vessels within the tissue section. Thereafter, the percentage of parkin-positive vessels, with or without CAA, was assessed in the subsequent serial section. Quantification was performed by both M.M.M.W. and J.G.J.M.B. (blinded). Both quantifications were averaged and level of significance of the percentage of parkin-positive vessels between control, AD and AD brain with CAA, was analyzed using a One-way ANOVA followed by a Tukey’s Multiple Comparison Test (analyzed by GraphPad Prism, version 4.03).

Astrocyte cell line treatment and quantitative PCR

To detect parkin mRNA expression in astrocytes under oxidative stress and in the presence of Aβ1-42, U373 cells were plated in 24-wells plates (Greiner Bio-One, Frickenhausen, Germany) and treated for 24 hr with 0, 100 and 500 µM tert-butyl hydroperoxide (tBH₂O₂, Sigma, Vienna, Austria) or 50 µM Aβ1-42 (Innovagen, Lund, Sweden) at 37°C in a 5% CO₂ incubator. Cell viability was not affected by these treatments (data not shown). After incubation, cells were washed twice with ice cold PBS and messenger RNA was isolated using the mRNA capture kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. cDNA was synthesized with the Reverse Transcription System kit (Promega, Madison, WI, USA) following manufacturer’s guidelines. Quantitative PCR (qPCR) reactions were performed in an ABI7900HT sequence detection system using the SYBR Green method (Applied Biosystems, Foster City, CA, USA) as described previously. 28 The following primers were used to detect expression levels of human parkin mRNA (forward, 5’-GGC TGG CTG TCA TTC TGC ACA C-3’; reverse, 5’-TCC CGG CTG CAC TCT TTG-3’) and GAPDH (forward, 5’-AGG TCA TCC CTG AGC TGA ACA G-3’; reverse, 5’-CGC CTG CTT CAC CAC CTT CTT G-3’). Obtained expression levels of parkin transcripts were normalized to GAPDH expression levels. All oligonucleotides were synthesized by Ocimum Biosolutions.
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(Ocimum Biosolutions, IJsselstein, the Netherlands). Statistical analysis was performed using One-way ANOVA followed by students’ t-test (analyzed by GraphPad Prism, version 4.03).

Results

Distribution of parkin in neocortex and white matter of control brain

In the grey matter of the neocortex, parkin immunoreactivity was predominantly observed in neuronal cell bodies and in a subset of glial cells (Figure 1A). In the white matter, parkin was observed in cells with an astrocyte-like morphology (Figure 1B). Moreover, in both white and grey matter, parkin was found in a subset of larger parenchymal and leptomeningeal vessels, but absent from capillaries (Figure 1A, B). Non-specific binding of secondary antibody was absent, as shown after omission of primary antibody (Figure 1C).

Parkin distribution in AD brain

In AD brains, the general staining pattern of parkin was similar to control brain. Parkin sporadically colocalized with classic SPs (Figure 2A-C), whereas parkin staining was absent in both diffuse SPs and diffuse Ab deposition surrounding vessels, the so-called dyshoric angiopathy (Figure 2G-I). In addition, no colocalization of parkin staining was observed with hyperphosphorylated tau in NFTs (Figure 2D-F). Interestingly however, all Ab-affected vessels in AD brain displayed marked parkin immunoreactivity (Figure 2G-I). In fact, quantitative analysis demonstrated a significant increase in parkin-positive vessels.
Parkin colocalizes with AD & MS lesions

in CAA-affected AD brains compared to control brain ($p>0.0001$) and AD brain without CAA ($p>0.0001$) (Figure 2K). Furthermore, parkin staining was observed in cells with an astrocyte morphology associated with SPs and CAA (Figure 2G-I). This was confirmed by co-localization of parkin with glial fibrillary acidic protein (GFAP), an astrocytic marker (Figure 2J).

**Parkin distribution in MS brain**

In active lesions, which are characterized by myelin loss (PLP staining, Figure 3A) and abundant inflammatory cells (MHC-II staining, Figure 3B), increased parkin immunoreactivity was observed (Figure 3D) compared to adjacent normal appearing white matter (NAWM) (Figure 3C). Parkin was found most predominant in astrocyte-like cells in active lesions (arrow, Figure 3D). In chronic active (CA) lesions (Figure 3E), which are composed of a hypocellular center and an inflammatory rim (Figure 3F), increased parkin immunoreactivity was observed compared to NAWM, particularly in the inflammatory rim (Figure 3G). This is in contrast to the hypocellular center, in which astrocytes were only weakly stained (Figure 3H). As in active lesions (Figure 3D), astrocytes seemed to be the predominant parkin expressing cell type in the inflammatory rim of CA lesions (arrows, Figure 3G). Chronic inactive (CIA) lesions had a similar Parkin staining pattern (data not shown) as the hypocellular center of CA lesions. Colocalization studies of parkin (Figure 3I) and GFAP (Figure 3J) confirmed increased immunoreactivity of parkin in astrocytes in inflammatory demyelinated areas (Figure 3K).

**Parkin mRNA expression is upregulated upon exposure to free radicals**

In order to evaluate whether parkin mRNA expression in astrocytes is upregulated upon oxidative stress, U373 cells were treated with 100 and 500 µM tbH$_2$O$_2$ for 24 hr. Treatment of U373 cells with both concentrations of tbH$_2$O$_2$ lead to a significant increase in the expression of parkin mRNA (100µM vs. control, $p\leq0.05$; 500µM vs control, $p\leq0.01$) (Figure 4). In contrast, treatment of the cells with 50 µM Aβ$_{1-42}$ for 24 hours did not alter parkin mRNA expression (data not shown).

**Discussion**

In the adult human and mouse brain, parkin is present in neuronal cell bodies and processes throughout the brain, as well as in a subset of glial cells. Tamo and coworkers showed that parkin is also observed in walls of leptomeningeal vessels, of which both endothelial and smooth muscle cells are intensely stained. In intraparenchymal vessels parkin staining is less intense, and parkin is absent from capillaries. In PD patients, parkin is found in neurons of the putamen, substantia nigra (SN) and frontal lobe cortex, whereas glial cells are only weakly immunopositive. In affected dopaminergic neurons in PD brains, parkin colocalizes with Lewy bodies, which are characterized by deposition of aggregated proteins.

Here, for the first time we demonstrate colocalization of parkin with characteristic AD and MS lesions. In AD brains, parkin was found to be associated with aggregated Ab deposits in classic SPs and CAA-affected vessels, and reactive astrocytes surrounding both SPs and amyloid-laden vessels. Similarly, astrocytes in inflammatory MS lesions displayed increased parkin immunoreactivity compared to astrocytes in the surrounding NAWM. In chronic inactive MS lesions parkin staining was only moderately increased in astrocytes.
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Figure 2. Double immunofluorescence staining of parkin (green) with Aβ (red) in SPs and CAA, and with hyperphosphorylated tau (red) in NFTs. Parkin was present in an occasional classic SP (A-C, arrows). No immunoreactivity of parkin was observed in NFTs (D-F, arrows). Parkin staining colocalized with Aβ in CAA affected vessels, and was observed in reactive astrocytes associated with CAA (G-I, arrows). Co-localization of parkin (red) with astrocytes surrounding parkin-laden CAA vessels was confirmed by double staining with GFAP (green) (J). The percentage of parkin-positive vessels was significantly increased in AD patients with CAA, compared to AD brains without CAA and control brain (K).

Figure 3. Immunohistochemical staining of parkin in active and chronic active MS lesions. Serial sections A–D, E–H. Active MS lesions are characterized by loss of PLP (A) and abundant MHC class II positive cells (B). In normal appearing white matter the anti-parkin antibody stained a subset of glial cells (C), whereas in active lesions staining was more intense in reactive astrocytes (D, arrows). Chronic active (CA) MS lesions are characterized by loss of PLP (E), abundant MHC class II positive cells at the edge of the lesions and only a few positive microglia in the center (F). Parkin immunoreactivity at the edge of the CA lesion was predominantly observed in reactive astrocytes (G, arrows), whereas the center showed a more diffuse staining pattern (H). Double immunofluorescent staining for parkin (I) and GFAP (J) showed co-localization of parkin with astrocytes (K). Original magnification: A–B 25×; E–F 50×; C–D, G–K 630×. Abbreviations: PLP = proteolipid protein, MHC = major histocompatibility complex, GFAP = glial fibrillary acidic protein.
Although the role of parkin in classic SPs, CAA and reactive astrocytes associated with both AD and MS lesions is currently unknown, parkin is suggested to protect cells against various stressors, including oxidative and unfolded protein-mediated stress. Therefore, we hypothesized that oxidative stress, characteristic of both AD and MS pathogenesis, could lead to induction of parkin expression. Indeed, parkin mRNA expression was found to be upregulated in astrocytoma cells upon exposure to free radicals. This indicates that parkin expression is upregulated in astrocytes in AD and MS upon oxidative stress and might counteract unfolded-protein stress and oxidative damage.

A possible role for parkin in AD pathogenesis has been suggested since in parkin-deficient mice, skeletal muscle cells demonstrated to be more vulnerable for intracellular Ab accumulation compared to controls. In addition, a recent study by Burns and coworkers found that parkin promotes ubiquitination and proteasomal degradation of intracellular Ab, thereby having a protective effect. Our data also indicate a possible role for parkin in AD, since we observed that parkin is associated with typical AD lesions in the human brain. We found that parkin is more associated with fibrillar Ab deposits, as observed in CAA and classic SPs, than non-fibrillar Ab deposits, predominantly observed in diffuse SPs. However, it remains unclear why parkin was only found in a subset of classic SPs, whereas all observed CAA-affected vessels demonstrated parkin immunoreactivity. This discrepancy might originate from the parkin levels within cells associated with these lesions, since vascular cells, such as endothelial cells and smooth muscle cells express relatively high levels of parkin. In addition, the preference of parkin for aggregated protein deposits has been observed in other studies. For instance, we recently demonstrated that parkin not only colocalizes with aggregated protein deposits in Lewy bodies, but also in corpora

Figure 3 continued.
Parkin colocalizes with AD & MS lesions

Whether parkin plays a role in the formation and/or functionality of these aggregated protein deposits remains to be elucidated. In brain, Ab is produced by neurons and transported towards the cerebral vessels via the interstitial fluid drainage. This results in increasing levels of Ab taken up by the (peri)vascular cells and subsequent formation of toxic free radicals. This process might be counteracted by activating or upregulating parkin as a virtue of protecting cells against cell damage. On the other hand, accumulation of intracellular Ab is also known to have an effect on the unfolded protein response, upon which parkin might be activated and/or upregulated. However, our data showed that Ab did not lead to increased parkin mRNA levels, despite the toxic Ab levels. Therefore, it is likely that only intracellular produced Ab is able to induce parkin upregulation, as demonstrated previously. Thus, although extracellular Ab might not itself activate astrocytes and possibly induce parkin expression, other Ab-deposit associated proteins, which are known to activate astrocytes to a larger extent as Ab, might. Remarkably, no upregulation of parkin levels was observed in umbilical vein endothelial cells in which unfolded protein stress was induced. Thus, although the origin of parkin observed in CAA and the processes underlying the association of parkin with CAA are still unclear, it is likely to reflect a protective response considering parkins’ alleged involvement in protective cellular mechanisms.

Several studies have shown that inflammation is an important mediator of cellular damage in MS. Infiltrated immune cells produce nitric oxide and reactive oxygen species, which are known to cause protein misfolding and oxidative stress and damage. Here, we demonstrate enhanced astrocytic parkin immunoreactivity in active inflammatory MS lesions. Interestingly, we also observed increased astrocytic parkin immunoreactivity in inactive lesions where inflammation has subsided. However, demyelinated axons and astrocytes in chronic lesions are still exposed to oxidative stress, as indicated by increased mitochondrial heat shock protein 70 expression.

Evidence is emerging that astrocytes play a dual role during neuroinflammation and neurodegeneration. Astrocytes are capable of enhancing immune responses by production of proinflammatory mediators and inhibiting myelin repair. On the other hand, astrocytes are able to suppress inflammation and sequester excessive extracellular glutamate. The beneficial role of astrocytes is further emphasized by their increased expression of antioxidant enzymes in MS and AD. In MS lesions as well as SPs and CAA in AD, astrocytes become reactive in response to changes in their environment. Reactive astrocytes might have an intrinsic increased parkin expression, or alternatively, the proinflammatory environment in MS and AD lesions might also influence parkin production by astrocytes. It has been shown that unfolded-protein stress leads to increased parkin protein levels in rat astrocytes. As both unfolded-protein and oxidative stress occur in...
AD and inflammatory MS lesions, it is likely that astrocytic parkin is upregulated by these stressors. Taken together, astrocytes appear to express an arsenal of protective proteins, including parkin, to defend themselves and surrounding tissue against inflammation and Aβ-induced cellular damage.

Of note however, besides parkin expression, total parkin activity is also influenced by posttranslational modifications of parkin. Although parkin is generally considered protective against nitrosative and oxidative stress, it has been reported that S-nitrosylation and oxidation of parkin results in reduced solubility of parkin, which will lead to loss of parkins’ neuroprotective ability. As described above, nitrosative and oxidative stress are common features of both AD and MS. Therefore, it is not unlikely that parkins’ activity is reduced in AD and MS.

In conclusion, we observed enhanced parkin immunoreactivity in AD and MS brain tissue. Parkin colocalizes with an occasional classic SP and with CAA-affected vessels in AD tissue as well as astrocytes associated with both lesions. Similarly, astrocytic parkin immunoreactivity was enhanced in demyelinated MS lesions. Moreover, parkin mRNA expression was found to be induced by oxidative stress in astrocytoma cells. As parkin is known to protect cells from various forms of stress, including unfolded protein, mitochondrial and oxidative stress, we speculate that enhanced parkin expression is an endogenous defense mechanism to counteract cellular stress-induced damage. Future experiments are warranted to reveal parkins’ role in the pathogenesis of neurodegenerative and neuroinflammatory diseases, and whether parkin might represent a therapeutic target for these neurological disorders.

Reference List


Parkin colocalizes with AD & MS lesions


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#### Tables

**Table 1. Summary of AD patient details**

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AD = Alzheimer's disease, CAA = cerebral amyloid angiopathy, F = Female, M = Male, PMI = Post Mortem interval (h = hours), ND = not determined, NFT = neurofibrillary tangles, Aβ = amyloid-beta. Grading of AD (Braak) and of CAA was performed as described in the materials and methods section.

**Table 2. Summary of MS patient details**

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MS = multiple sclerosis, F = Female, M = Male, PMI = Post Mortem interval (h = hours), RR = relapsing remitting, SP = secondary progressive, PP = primary progressive, ND = not determined, A = active lesion, CA = chronic active lesion, CIA = chronic inactive lesion. Classification of MS lesion type was performed as described in materials and methods section.