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RESEARCH ARTICLE

Distribution of parvalbumin and calretinin immunoreactive interneurons in motor cortex from multiple sclerosis post-mortem tissue

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Abstract Parvalbumin (PV) and calretinin (CR) are calcium binding proteins (CBP's) expressed in discrete GABAergic interneuron populations in the human cortex. CBP's are known to buffer calcium concentrations and protect neurons from increases in intracellular calcium. Perturbations in intracellular calcium can activate proteolytic enzymes including calpain, leading to deleterious effects to axons. Ca++-mediated mechanisms have been found to be associated with axonal pathology in MS and the restructuring of calcium channels has been shown to occur in experimental autoimmune encephalomyelitis (EAE) as well as multiple sclerosis tissue. Previous data indicates a reduction in the expression of the parvalbumin gene as well as reduced extension of neurites on parvalbumin expressing interneurons within multiple sclerosis normal appearing grey matter (NAGM). Modifications in interneuron parvalbumin or calretinin levels could change calcium buffering capacity, as well as the way these cells respond to neuronal insults. The present study was designed to compare CBP immunoreactive neurons in normal and multiple sclerosis post-mortem NAGM. To this end, we utilized immunofluorescent staining and high resolution confocal microscopy to map regions of the human motor cortex, and characterize layer specific CBP distribution in the normal and multiple sclerosis motor cortex. Our results indicate a significant reduction in the number of PV interneurons within layer 2 of the multiple sclerosis primary motor cortex with no concurrent change in number of calretinin positive neurons.

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Introduction

Multiple sclerosis is an autoimmune demyelinating disease characterized by focal inflammation, loss of myelin, neuronal dysfunction and degeneration. In addition, it has recently been documented that reductions in the expression of neuronal proteins in the cortex and loss of grey matter volume occur early in the disease (Chard et al. 2002) and these cortical changes have been observed in normal appearing grey matter (NAGM). Specifically, we have noted significant reductions in the expression of several genes important in GABAergic neurotransmission, particularly, glutamate decarboxylase-67 (GAD 67) and several of the GABA receptor subunits (α1 and γ3) in MS motor cortex (Dutta et al. 2006). We are particularly interested in GABAergic changes, since these inhibitory interneurons have been proposed to be important in the synchronization and rhythmic control of motor neuron output (Porter et al. 2000). GABAergic interneurons can be categorized based on differential staining for the calcium binding proteins parvalbumin (PV), calretinin (CR) and/or calbindin (CB) (DeFelipe 1997; del Rio and DeFelipe 1997; Grateron et al. 2003; Cotter et al. 2002). PV, CB and CR are all members of the calcium binding protein EF-hand super family and are important in buffering intracellular calcium (Baldellon et al. 1998). Parvalbumin is expressed in a subset of GABAergic inhibitory interneurons and PV-staining cells generally display large basket or chandelier cell morphologies and have been classified as fast spiking interneurons (Amitai et al. 2002). Calretinin stains a different subset of GABAergic interneurons that are generally bipolar, Cajal-Retzius or

double bouquet type cells (DeFelipe 1997). Of significance to neurological disease, perturbations in CBP's have been suggested to accompany a number of pathological conditions (Beers et al. 2001; Eyels et al. 2002) including multiple sclerosis. Parvalbumin is known to buffer calcium and has been shown to protect neurons from excess intracellular calcium (Beers et al. 2001; Dekkers et al. 2004). Restructuring of calcium channels has been documented in EAE and MS tissue (Craner et al. 2004; Kornek et al. 2001) and these changes may lead to perturbations in neuronal calcium levels resulting in deleterious effects on neurons (Dutta et al. 2006). Interestingly, PV expression has been reported to be significantly diminished in NAGM from MS brain (Dutta et al. 2006). However, accompanying alterations in neuronal morphology, changes in specific cortical layers, or involvement of other CBP's have not been reported. Therefore, the present study was designed to examine and compare the laminar distribution of PV- and CR- containing neurons in normal and MS NAGM. We investigated changes at the level of the motor cortex and suggest its potential involvement in axonal dysfunction, as well as, grey matter degeneration. We propose, that in addition to our reported mitochondrial changes in MS cortex (Dutta et al. 2006), the expression of an important calcium binding protein, PV (but not CR), is markedly reduced in normal appearing grey matter of MS motor cortex.

Materials and methods

Human tissue

Formalin fixed control and MS tissue blocks were obtained from primary motor cortex. Control tissue was donated by Kathleen Price Bryan Brain Bank and the MS tissue donated by Rocky Mountain MS Center. Cortical tissue was cut on a media cooled vibratome into 40 or 50 μm slices. Tissue sections were subjected to microwave-induced antigen retrieval utilizing antigen unmasking solution (Vector Labs, Burlingame, CA) for 21 min on high power (3 × 7 min). Sections were then incubated for 1 h in PBS containing 0.5% triton x-100 and normal donkey serum to block non specific binding, and washed 3 times 10 min in PBS. Free-floating sections were incubated with monoclonal mouse anti-parvalbumin (1:1,000, SWANT, Switzerland) or goat anti-calretinin (1:500, Chemicon, Temecula, CA) and rabbit anti-neurofilament (1:1,000 Chemicon, Temecula, CA) antibodies for 18 h. After 3 × 10 min washes the sections were incubated for 6 h in donkey anti-mouse alexa fluor 488 or donkey anti-goat alexa fluor 488 (1:300), donkey anti-rabbit alexa fluor 555 IgGs (1:300) and Topro-3 (1:1,000, Molecular Probes). After three washes, sections were mounted and dried on coated slides followed by 10 min staining with sudan black b (Sigma Aldrich) in 70% ethanol to mask lipofuscin autoflourescence, and finally coverslipped with Vectashield antifade reagent (Vector Labs, Burlingame, CA). Adjacent sections were also stained for proteolipid protein, macrophage infiltrates, astrogliosis and sudan black b to confirm the absence of grey matter lesions in the selected tissue specimens.

Scanning and analysis

An Olympus FV 500 confocal microscope equipped with four lasers and a motorized XYZ stage was used to acquire the images. Only sections directly adjacent to those processed and confirmed to have no lesions were included in the study. Using the neurofilament stain as a guide (to ensure all layers of the cortex were included in analysis and no lesions were included) four contiguous 10× objective fields were imaged from the outer edge of the cortex to the white matter within each sample. For each field, an image stack of optical sections (approximately 50) spanning the entire depth of the tissue section was acquired. Imaging software (ImageJ, NIH) was used to recombine the fields into a single cortical map of the gray matter. Image stacks were examined for stacked cells to ensure no cells were missed in the counting step. After a maximum intensity projection was performed on the montaged image stack, cells were counted with ImageJ's cell counting plugin. Counts from the 40 μm sections were divided by .8 to normalize the data to the 50 μm samples. Preliminary experiments were performed to confirm that the antibody would fully penetrate all the way through the tissue in both the 40 and 50 μm sections. To ensure penetration, free floating sections were used so the antibody could penetrate from both sides of the section; a maximum of 25 μm to the inside of the tissue. We also used long antibody incubation times to maximize antibody diffusion and binding. In addition, image stacks were inspected for brightness inhomogeneities in the middle of the section, since incomplete antibody penetration would be accompanied by a reduction in antibody binding within the central region of the section. We saw no reduction in intensity, even when using 75 μm thick sections with our protocol indicating that antibody penetration was complete. The use of neurofilament stain permitted the clear visualization of the laminar nature of the cortex. Only regions containing all layers of the cortex and having neural fibers streaming perpendicular from the pial surface to the white matter were scanned. This ensured that all layers of the cortex were included in each acquired dataset, and that the tissue slice was perpendicular to the outer surface of the cortex. After all cellular counts had been performed, acquired data were aligned horizontally (using neurofilament fluorescence) and a digital line drawn across

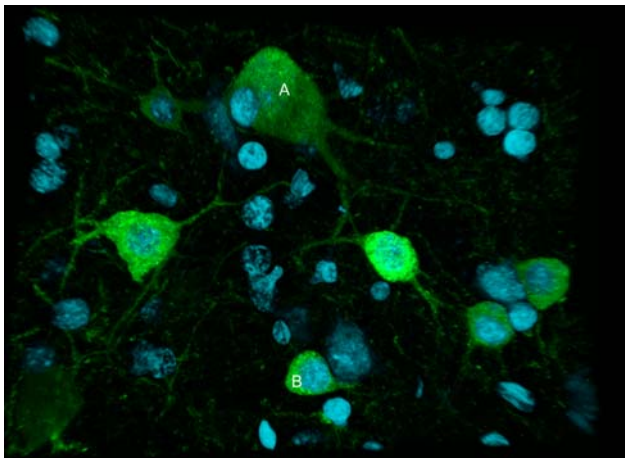

Fig. 1 This confocal micrograph displays the typical parvalbumin staining patterns observed in the current study. This data is from a control brain section. Parvalbumin positive cells generally appeared as having large basket (**a**) or chandelier (**b**) cellular morphologies most easily defined based on somal size. Parvalbumin is colored *green* and nuclei (Topro-3) are *light blue*

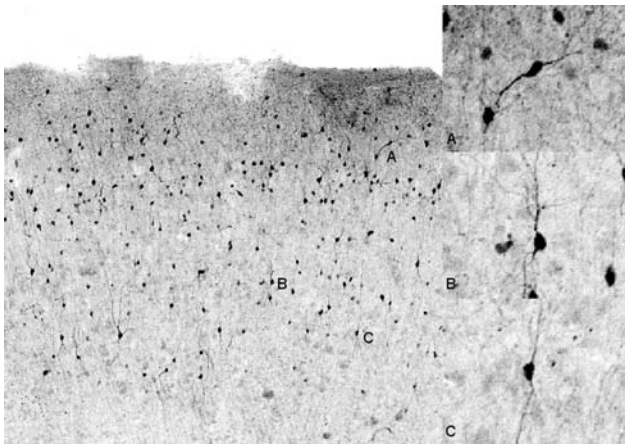

Fig. 2 These images represent typical calretinin positive staining patterns observed in the current study. Cells were mainly limited to layers 1–3 of the cortex, and no statistically significant differences were observed between groups. CR-positive cells typically displayed **a** Cajal-Retzius, **b** double bouquet or **c** bipolar type cellular morphologies most easily identified by number and orientation of neuronal projections. This micrograph was generated from an MS tissue section

all datasets. Lines were drawn to delineate the edges of the cortex layers across all samples. The number of PV and CR positive cells within each delineated layer was analyzed and compared. Data was analyzed using ANOVA in Microsoft Excel, and a P value of <0.05 considered significant.

Results

Demographic data describing the brain age, post mortem interval (PMI) and disease state (where applicable) for each of the post mortem samples is presented in Table 1. Attempts were made to control for sex, age and PMI where possible. The fluorescent staining procedure clearly permitted the visualization of PV- and CR-positive interneurons within the cortical gray matter. PV-positive cells could be morphologically classified based on somal size and neuronal projections. Generally, the cells were of large basket or chandelier type morphologies, as previously reported in the literature (Fig. 1). Calretinin-positive cells were more difficult to characterize but generally displayed Cajal-Retzius, double bouquet type or bipolar morphologies (Fig. 2 a–c) as reported in the literature. Utilizing neurofilament staining as a guide, it was apparent that PV containing cells were mainly located in layers 2–4 of the motor cortex within the montaged cortical maps (Fig. 3), whereas CR immunoreactivity was mainly limited to layers 1–3 (Fig. 2). Utilizing the cell counting software, the total number of PV- and CR-positive cells were documented and are represented in Fig. 4a, b. The total number of PV-immunopositive cells throughout the cortical sections was reduced by 40% in MS tissue compared to controls. In order to determine whether these

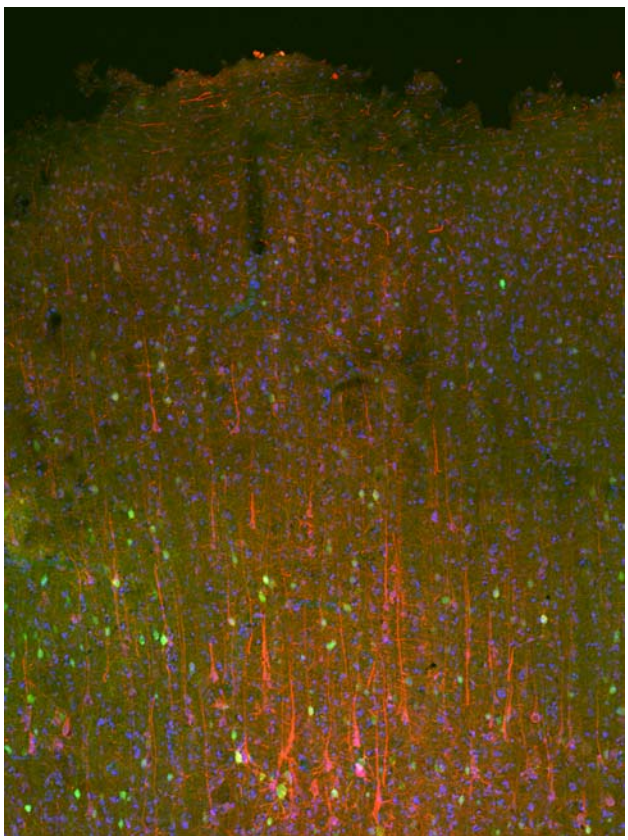

Fig. 3 This image is a three channel confocal micrograph representative of the parvalbumin data generated in the present study from a control section. Parvalbumin cells are colored *green*, neurofilament *red* and nuclei *blue*. Multiple 10× microscopic fields were captured and merged to generate high resolution maps of the cortex to assess the laminar distribution of parvalbumin immunoreactive cells

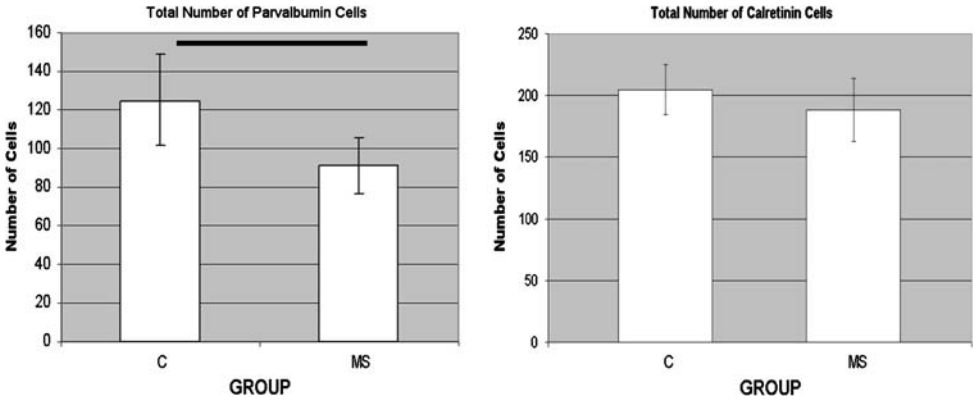

Fig. 4 **a**, **b** These figures represent the comparison of CBP-positive cells from multiple sclerosis and control NAGM. Control NAGM contained significantly more total immunoreactive PV cells, while no differences were observed in total number of CR positive cells between the two groups. Significance is denoted by a *solid black line*, and a *P* value of <0.05 considered significant

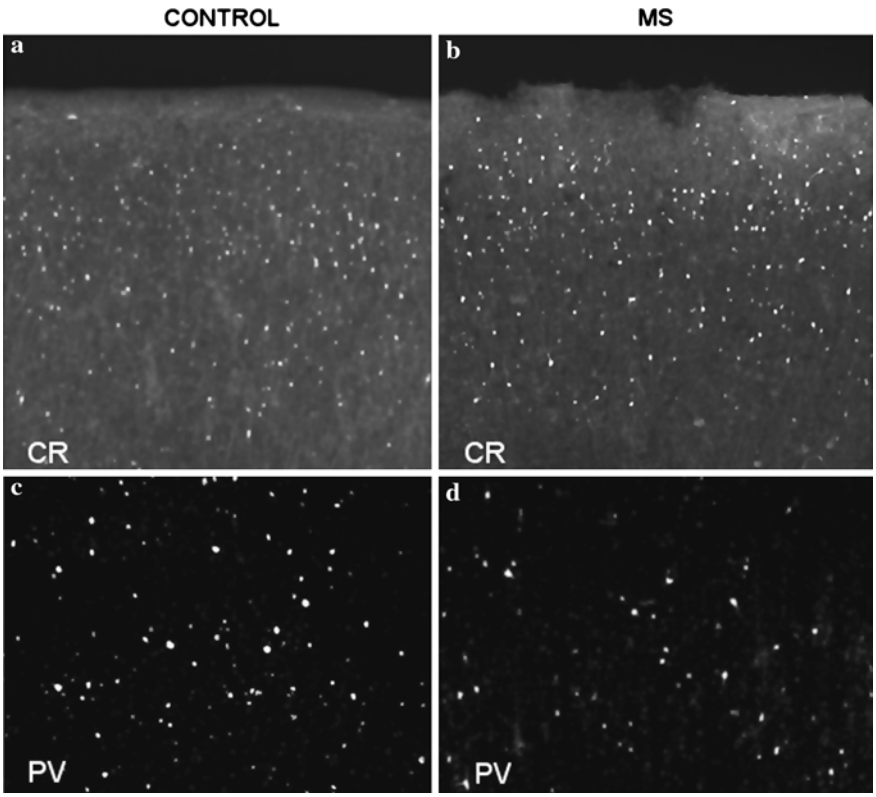

Fig. 5 These micrographs compare control and MS CBP staining in the motor cortex. The controls are on the *left* and MS on the *right*. No difference was observed between cell numbers in the calretinin samples (**a**, **b**), however a significant reduction in parvalbumin immunoreactivity was observed in layer 2 of the MS cortex as displayed by comparative micrographs from this region of the primary motor cortex (**c**, **d**)

differences were merely a consequence of age, PMI or fixation, we counted numbers of other GABAergic interneuron populations, specifically those containing CR. In contrast to PV-immunopositive cells, there was no difference in numbers of CR-immunopositive neurons between MS and control slices (Fig. 5a, b). Also, there was no significant correlation between PMI and numbers of PV- or CR-stained cells in MS or control slices. Numbers of CR-immunopositive cells were similarly not affected by age in either group. Conversely, there appeared to be a significant relationship between age and number of PV-immunopositive cells in MS cortex but not in the controls (Fig. 6).

To further characterize the differences in PV-immunopositive cell numbers we examined the laminar distribution to determine whether certain layers expressed more or less cellular loss. Interestingly, the reduction in PV-staining cells noted in the total count appeared to be as a result of a specific loss of these neurons in layer 2 of the cortex, since the number of PV-immunopositive cells in this layer was reduced by 40% (Fig. 5c, d). There was also a 25% decrease in the numbers of PV-immunopositive cells in layer 3, however, this difference did not reach statistical significance. There were no differences detected in layer 4 (Fig. 7).

Discussion

The current study reveals a significant loss of PV-containing interneurons in MS NAGM from primary motor cortex

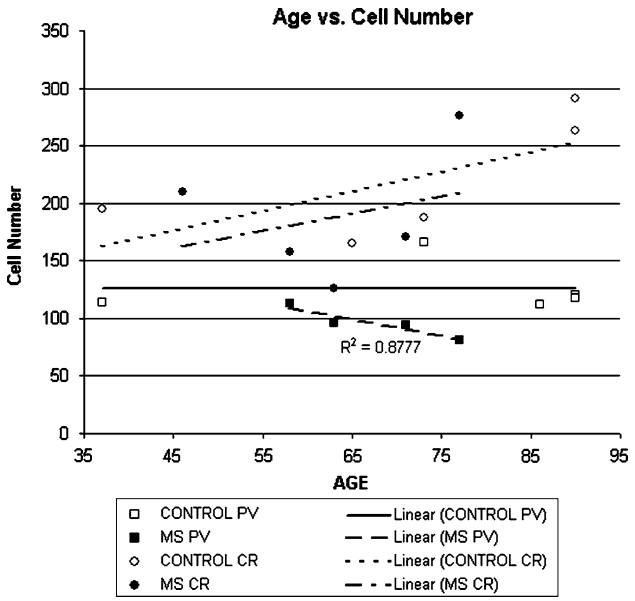

Fig. 6 This figure displays age versus cell number for all the samples used in the current study. Regression analysis indicates a significant correlation with age and PV cell number in MS patients. No correlation was observed in controls suggesting that PV expression is selectively reduced with long term MS disease course

Table 1 This table displays the data from each of the post-mortem samples used

	Age	PMI (min)	Sex	Staining	Disease type
Control					
995	90	210	F	PV/CR	
571	37	339	M	PV/CR	
849	90	225	F	PV/CR	
855	73	97	F	PV/CR	
566	65	331	M	CR	
45	86		F	PV	
MS					
160		1,440	F	PV	Chronic progressive
216	71	270	F	PV/CR	Chornic inactive
220	63	2,880	F	PV/CR	Chornic inactive
223	58	345	F	PV/CR	Chornic inactive
219	46	240	F	CR	Chornic active
221	77		M	PV/CR	Chornic inactive

Post mortem interval, age of death and MS disease state (where applicable) is noted. Blank area indicates unknown data and staining indicates whether the samples were processed for PV and/or CR immunoreactivity

when compared to controls, and this change was not accompanied by any modifications in number of CR-positive cells regardless of age or PMI. Further, this reduction in detectable immunoreactive PV-containing interneurons is particularly significant in layer 2 of the cortex and represents the layer with the largest reduction in this specific interneuron type. The reasons for these differences in PV interneuron cell numbers are not currently known, however, these findings are consistent with the reported reduction in GAD67 and PV mRNA levels and protein expression in our previous study (Dutta et al. 2006). We also noted in this study a correlation between the numbers of PV-staining cells and age in the MS group only. This may suggest a link between disease duration and loss of these particular GABAergic interneurons. Taken together, it appears that there is a selective reduction in PV-containing, GABAergic interneurons in NAGM from MS cortex.

The impact of losing these inhibitory interneurons on MS are currently unknown. However, the consequences of reductions in PV may render these neurons susceptible to perturbations in calcium homeostasis that could potentially lead to their degeneration due to several factors. Calcium when unchecked, increases intra-axonal calcium levels, can cause protease activation, mitochondrial dysfunction and increases in reactive oxygen species that can lead to deleterious effects on neurons (Chard et al. 2002; Rosin et al. 2004). A disruption in calcium homeostasis, perhaps through excitotoxicity, has been suggested to occur in MS,

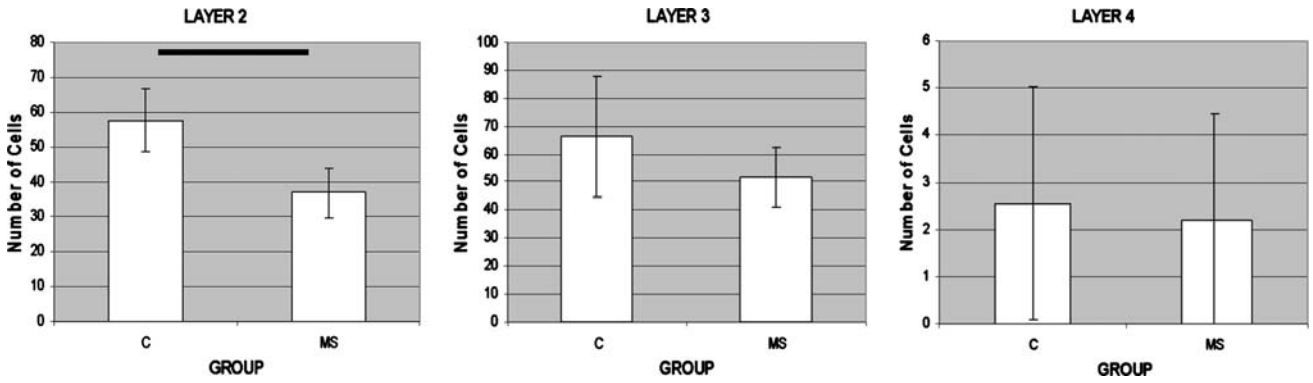

Fig. 7 To further elucidate specific regional changes in PV neurons, cellular distribution was assessed by layer. It is apparent that significantly fewer parvalbumin positive cells exist within layer 2 of the MS cortex, with no other layer based differences. Significance is denoted by a *solid black line*, and a *P* value of <0.05 considered significant

and calcium channel blockers are known to ameliorate symptoms in a mouse model of multiple sclerosis (Brand-Scheiber and Werner 2004). It has also been demonstrated that PV expression protects motoneurons from sustained increases in intraneuronal calcium associated with amyotrophic lateral sclerosis (Beers et al. 2001) as measured by increases in neuronal survival.

A decrease in PV-containing inhibitory interneurons could perturb normal neuronal function in the cortex. It has been suggested that loss of these neurons may result in asynchronous, uncoordinated neuronal activity within the motor cortex. In addition, dysfunction or loss of GABAergic interneurons may potentially result in dysinhibition of glutamatergic neuronal activity. There is ample evidence indicating that excitotoxic damage partially underlies neurological deficits associated with MS (Gilgun-Sherki et al. 2004). Indeed, glutamate can be toxic to both oligodendrocytes and neurons alike (Dutta et al. 2006; Ibrahim et al. 2001); Isaev et al. 2005), and glutamate levels, as well as cortical excitability, are known to increase in MS tissues (Srinivasan et al. 2005). The impact that the loss of these neurons may have on cortical function is further supported by studies utilizing transcranial magnetic stimulation (TMS) in MS patients (Caramia et al. 2004; Liepert et al. 2005). These findings suggest that during the relapsing phase of the disease, or in MS patients experiencing fatigue, there is an association with cortical hyperexcitability. Both authors implicate dysinhibition and an imbalance between GABA/glutamate transmission in cortical networks, as a possible cause. Our results support this hypothesis, and suggest that specific populations of GABAergic interneurons are selectively affected by MS, that could potentially cause these observed effects on cortical excitability and inhibition.

Methodologically, it should be noted that the post-mortem interval (PMI) for the MS sample group was significantly longer than that for the control brains. PMI, as well as formalin fixation time, can have effects on antibody binding and consequently immunohistochemical results. In the current study, differences were only observed in PV-positive neurons, and not CR-positive cells. Further, if this difference was due to a longer PMI or fixation time it would be logical to expect that both interneuron subtypes would be similarly affected. It does remain possible that CR immunofluorescence is differentially susceptible to these parameters. However, regression analysis indicated no correlation with PMI and cell number in either the CR or PV studies indicating these parameters were not a factor in the current results. Age related reductions in CBP immunoreactivity (Bu et al. 2003) have also been reported in human cortex. These differences appear to be limited to CR and calbindin (not PV) and were not apparent in primary motor cortex so should not impact the observed differences in the current study. It should be re-iterated that regression analysis suggested a negative correlation between age and MS PV number, a correlation that was not apparent in control tissue. In fact, this was the only observed correlation, with no correlation being apparent with PMI/fixation time and CR/PV cell numbers. Our current observations indicate that PV-containing GABAergic interneurons are affected by MS, however, CR-positive interneurons are seemingly unchanged.

The reason for this selective vulnerability is currently unknown, but could be associated with differences in PV and CR neuronal development, structure and/or function. Developmentally, in some regions of human cortex it has been shown that PV immunoreactivity only appears postnatally (Grateron et al. 2003), while CR and CB immunoreactivity is already apparent at birth. Electrophysiologically, PV containing interneurons have been labeled as fast spiking interneurons with a high metabolic demand, while CR immunoreactive neurons in primate cortex have been shown to have characteristics of non-fast spiking cells (Zaitsev et al. 2005). Structurally, PV interneurons are known to form electrically and chemically coupled syncitia, as well as synapse on pyramidal cells (Amitai et al. 2002; DeFelipe 1997). CR neurons are known to form symmetrical and asymmetrical synapses with other interneurons (PV and CR immunoreactive) as well as pyramidal cells in human cortex (DeFelipe 1997). The specific function ascribed to individual GABAergic interneuronal populations is defined by morphological and physiological properties of the neurons. Subpopulation specific features may render PV-containing interneurons more vulnerable to changes in the microenvironment, dysfunction and/or degeneration. It is clear that differences in developmental, electrophysiological, as well as structural parameters exist between theses two interneuron populations, and it will be of importance to determine why these two populations are differentially susceptible in MS. Further, it will be useful to determine if the changes are a secondary effect on neurons which are unable to respond to the physiological stresses associated with MS, or a primary defect in PV-containing interneurons that may be involved in the development of MS symptoms.

Loss of PV and other GABAergic interneurons (Cotter et al. 2002; Eyels et al. 2002), as well as, cortical hyperexcitability has been observed in other neurological diseases and this phenotype of neurons appears exceptionally vulnerable to stressful insults. Further studies should be designed to determine if these changes are directly linked to neurodegeneration within the cortex of MS tissue and whether they represent an initiating insult, or an effect of early neuronal dysfunction.

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